# Chapter 2

# Materials and Experimental procedures

## 2.1 Material

### 2.1.1 Chemicals, buffers

#### Chemicals

All reagents were obtained from Carl Roth (Karlsruhe, Germany) or Sigma-Aldrich (Taufkirchen, Germany) unless stated otherwise. The PKA-activity assay was obtained from Millipore (Schwalbach, Germany), restriction enzymes were obtained from New England Biolabs (Frankfurt a.M., Germany). Water (referred to as *A. bidest*) was purified by the Milli-Q Plus-system to $\leq 10\mu$S/cm at room temperature (RT).

#### Buffers

Buffers were generated as stock solutions and diluted to working concentration (1x) with *A. bidest* if not stated otherwise.

**Blot buffer (10x)**

**'semi dry buffer'**

| | |
|---|---|
| 58.2 g | Tris (48 mM) |
| 29.3 g | glycine (39 mM) |
| 0.38‰ | SDS (1.3 mM) |

ad 1 l with *A. bidest*

**Blot buffer (1x)**

**'semi dry buffer'**

| | |
|---|---|
| 100 ml | 10x blot buffer |
| 200 ml | methanol |

ad 1 l with *A. bidest*

**Lysis buffer**

| | |
|---|---|
| 1.14 g | $KH_2PO_4$ (10 mM) |
| 21.92 g | NaCl (150 mM) |

adjust to pH 7.4

| | |
|---|---|
| 1,46 g | EDTA (5 mM) |
| 1.90 g | EGTA (5 mM) |
| 1% (v/v) | Triton X-100 |
| 0.2% (w/v) | sodium deoxycholate |
| 1% (w/v) | Igepal CA-630 (NP-40) |
| 3.20 $\mu$g/ml | soybean trypsin inhibitor (STI) |
| 2.00 $\mu$g/ml | aprotinin |
| 0.12 $\mu$g/ml | benzamidine (1 mM) |
| 0.88 $\mu$g/ml | PMSF (0.5 mM) |

ad 500 ml with *A. bidest*

**SDS-sample buffer**

**('Lämmli buffer', 4 x)**

| | |
|---|---|
| 15 ml | glycerol |
| 7.5 ml | SDS (20% stock) |
| 25 ml | tris (0.625 M stock) |
| 1% (w/v) | bromphenol blue |

adjust to pH 6.8, ad 50 ml with *A. bidest*

| | |
|---|---|
| 30 $\mu$l | dithiothreitol (DTT, 1 M) |

Final concentration (1x) was obtained by adding the appropriate amount of Lämmli buffer to the given sample-volume.

**SDS-PAGE running buffer**

**(10x):**

| | |
|---|---|
| 30 g | tris (0.5 M) |
| 144 g | glycine (0.19 M) |

| | |
|---|---|
| 10 g | sodium dodecylsulfate (SDS, 35 mM) |

ad 1 l with *A. bidest*

**Phosphate-buffered saline (PBS, 10x)**

| | |
|---|---|
| 80 g | NaCl (274 mM) |
| 2 g | KCl (27 mM) |
| 2 g | $KH_2PO_4$ (15 mM) |
| 11.5 g | $Na_2HPO_4$ (81 mM) |

adjust to pH 7.4, ad 1 l with *A. bidest*, autoclave

**Separating gel buffer**

| | |
|---|---|
| 37.8 g | Tris (0.625 M) |

adjust to pH 6.8, ad 500 ml with *A. bidest*, autoclave

**Stacking gel buffer**

| | |
|---|---|
| 45.41 g | Tris (0.75 M) |

adjust to pH 8.8, ad 500 ml with *A. bidest*, autoclave

**Lysogeny broth (LB)-medium**

| | |
|---|---|
| 50 g | Pepton |
| 25 g | NaCl (17 mM) |
| 25 g | Yeast Extract |

adjust to pH 7.5, ad 5 l with *A. bidest*, autoclave

for dishes: 15 g/l agar

**Tris-acetate-EDTA buffer TAE (20x)**

| | |
|---|---|
| 96.8 g | Tris (1.6 M) |
| 22.84 ml | Acetic Acid (99-100%) |
| 5.84 g | EDTA (0,02 M) |

adjust to pH 8.0

ad 1l with *A. bidest*

**Tris-buffered saline with Tween-20 TBST (10x)**

| | |
|---|---|
| 6.06 g | Tris (10 mM) |
| 43.83 g | NaCl (150 mM) |

adjust to pH 7.4

| | |
|---|---|
| 5 ml | Tween-20 |

ad 1 l with *A. bidest*

### 2.1.2 Antibodies

**Primary antibodies**

| | |
|---|---|
| AQP2-antiserum H27 (rabbit) and | were described |
| Anti-GFP-antiserum (rabbit) | previously [98, 99] |
| Anti-GFP (mouse, monoclonal) | Clontech, USA |
| PKA-RII$\alpha$ (mouse, monoclonal) | BD Biosciences |

**Secondary antibodies**

Peroxidase-conjugated:

| | |
|---|---|
| goat anti-rabbit ($F(ab')_2$ fragments) | Dianova, Hamburg, Germany |
| rabbit anti-mouse ($F(ab')_2$ fragments) | Dianova, Hamburg, Germany |
| Cy3-conjugated goat anti-rabbit IgG | Jackson ImmunoResearch Laboratories, USA |

### 2.1.3 Peptides and proteins

**Peptides**

The AKAP-PKA disrupting peptides Ht31 (DLIEEAASRIVDAVIEQV KAAGAY), AKAP7$\delta$-wt-pep. (PEDAELVRLSKRLVENAVLKAVQQY), AKAP7$\delta$-L314E (PEDAELVRLSKRLVENAV<u>E</u>KAVQQY), the negative control peptides Ht31-P (DLIEEAASR<u>P</u>VDAV<u>P</u>EQVKAAGAY), AKAP7$\delta$-L308D (PEDAELVRLSKR<u>D</u>VENAVLKAVQQY), AKAP7$\delta$-PP (PEDAEL VRLSKRL<u>P</u>ENA<u>P</u>LKAVQQY) and N-terminal stearate- or biotin-coupled versions thereof were synthesised by the Peptide Synthesis group (Dr. Michael Beyermann, FMP). Peptides were synthesised to a purity > 90%,

provided lyophilised (see 2.2.6) and dissolved (10mM stock-solution) by dimethylsulphoxide (DMSO) and further dissolved to working concentrations with *A. bidest*, buffer or media, as indicated.

#### Proteins

Catalytic subunits of PKA were obtained in a concentration of 1.66 mg/ml (Promega, Mannheim, Germany). Purified recombinant, cAMP-free regulatory subunits of PKA (human RII$\alpha$: 0.75 mg/ml, rat RII$\beta$: 0.20 mg/ml) were a kind gift from the group of Prof. Dr. F. Herberg (Universität Kassel, Germany). Bovine RII subunits (2.7 mg/ml) were purchased from Sigma-Aldrich.

### 2.1.4 Bacterial hosts, eucaryotic cells, animals

#### Bacterial hosts

The *Escherichia coli (E. coli)* strain Top10$\beta$ was used for the amplification of plasmid DNA (Invitrogen, Karlsruhe, Germany).

#### Eucaryotic cells

Human embryonic kidney **(HEK293)** cells, human neuroblastoma **SH-SY5Y** cells and African green monkey kidney fibroblasts (COS7, Simian virus 40 transfected) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany. WT-10 cells (Madin-Darby canine kidney cells stably transfected with human AQP2 cDNA, under the control of cytomegalovirus promoter) were kindly provided by P.M. Deen [100]. Renal inner medullary collecting duct **(IMCD)** cells and rat **neonatal cardiac myocytes** were obtained as described below (see

2.2.7). All eucaryotic cells were grown at 37℃ in a humidified atmosphere of 95% air and 5% $CO_2$.

#### Animals

For the generation of primary cells and tissue-derived lysates rats were sacrificed by decapitation (*Rattus norvegicus*, strain: Wistar). Organs were preparated and immediately transferred to sterile PBS (4℃).

### 2.1.5 Apparatus and software

#### Apparatus

| | |
|---|---|
| Gel cast-stand and electrophoresis chamber | BIORAD Laboratories, München, Germany |
| Centrifuges | Beckmann TLK 100, Krefeld, Germany<br>Beckmann Optima L70<br>Sorvall RC 285, Bad Homburg, Germany<br>Haereus Biofuge pico, Osterode, Germany<br>Eppendorf MiniSpin, Hamburg, Germany |
| EPC-9 patch clamp amplifier | HEKA Elektronik, Lambrecht, Germany |
| J-720 spectrometer | Jasco, Groß-Umstadt, Germany |
| Lumi-Imager F1 | Roche Diagnostics, Mannheim, Germany |
| Microscopes | Zeiss 510 META (UV, NLO and FCS) inverted confocal laser-scanning microscope, Jena, Germany |

| | |
|---|---|
| Photometer | GeneQuantII, GE Healthcare, Freiburg, Germany |
| Pipettes | Eppendorf |
| Power supplies | Bio-Rad, Amersham Pharmacia Biotech |
| Rotator | Blood tube rotator SB1, Stuart Scientific, UK |
| Scintillator | Wallac 1409, Liquid Scintillation Counter PerkinElmer, Rodgau, Germany |
| Semi-dry Western Blot chamber | BIORAD |
| Sonicator | Sonoplus UW 2040, Bandelin Electronics Berlin, Germany |
| Thermomixer | Eppendorf Thermomixer 5436 |
| Pure-water generator MilliQ plus | Millipore |

**Software**

| | |
|---|---|
| AMBER 7.0 | Case *et al.* [101] |
| Axio Vision | Zeiss |
| bioperl v.1.4 | www.bioperl.org [102] |
| clustalw | www.ebi.ac.uk/clustalw [103] |
| Excel 2000 | Microsoft, Redmond, USA |
| GraphPad Prism 3.02 | GraphPad software, San Diego, USA |
| ImageQuant v5.1 | GE Healthcare |

| | |
|---|---|
| LaTeX MiKTeX-TeX 1.24.1 | D. E. Knuth |
| Lumi Analyst V 3.0.00.00 | Boeringer Mannheim |
| perl v5.8.3 | ActiveState Corp. |
| Photoshop 6.0 | Adobe |
| Powerpoint 2000 | Microsoft |
| PROCHECK | Laskowski *et al.* [104] |
| Prosite | www.expasy.org/prosite |
| Protein Data Bank (PDB) | www.rcsb.org/pdb [105] |
| PSIPRED | http://bioinf.cs.ucl.ac.uk/psipred [106] |
| Pulse software | HEKA Elektronik |
| Scansite | http://scansite.mit.edu [107] |
| SYBYL 6.91 | TRIPOS Inc., St. Louis, U.S.A. |
| TeXMAKER v2 | Pascal Brachet (www.xm1math.net/texmaker) |
| VectorNTI 10.0 | Invitrogen |
| VMD for WIN32, v. 1.8.5 | www.ks.uiuc.edu/Research/vmd [108] |
| Win-MAXC | www.stanford.edu/∼cpatton/winmaxc2.html |
| Winpep 1.22 | Lars Hennig [109] |
| Word 2000 | Microsoft |

## 2.2 Experimental procedures

Experimental procedures and standard methods without citations follow the methodology of Sambrook and Russell [110].

### 2.2.1 Molecular biology techniques

The isolation of RNA, generation of complementary DNA (cDNA) and molecular cloning was performed by Michael Gomoll (technical assistant, FMP). The yellow fluorescent AKAP7$\delta$-YFP (vector: pEYFP-N1, Clontech) construct was generated by Bayram Edemir in parallel to the cyan fluorescent protein (CFP) version described previously [71].

#### RNA isolation

RNA was isolated from SH-SY5Y cells and from rat brain tissue as starting material for the generation of human and rat CN129 clones (see below), each by utilising trizol reagent (Invitrogen) following the manufacturers instructions.

#### cDNA synthesis

RNA was reverse transcribed into cDNA by reverse transcriptase-polymerase chain reaction (PCR) using random hexamer oligo-nucleotides as starting points for DNA-synthesis, according to the instructions of the manufacturer (SuperScript First Strand Synthesis system for RT-PCR, Invitrogen). In order to generate a partial CN129 construct (amino acids: 1-126) the gene specific primers fw-CN129 human (5′-ATGCGCTAGCGATGGAAACAG ACTGTAATCCCATG-3′) and rv-CN129 prtl. human (5′-GCATACCGG TAGCAGTGCGTTTCCAAATGC-3′) were utilised in a PCR reaction

[cycles: 2 min 95℃, 24x (45 s 95℃, 45 s 62℃ - 0.5℃/cycle, 3.5 min 72℃), 20x (45 s 95℃, 45 s 50℃, 3.5 min 72℃) 5 min 72℃]. In order to generate the full length human and rat CN129 constructs (amino acids: 1-139) the gene specific primers fw-CN129 (human: 5′-ATGCGCTAGCTATGGAAA CAGACTGTAATCCCATG-3′, rat: 5′-ATGCGCTAGCTATGGAAACAG ACTGTAATCCCG-3′) and rv-CN129 f.l. (human: 5′-GCATACCGGTCC TGACTGTCCATCTCTTTTCAAAG-3′, rat: 5′-GCATACCGGTCCGGA CTGTCCATCTCGTTTC-3′) were utilised for PCR reaction: [cycles: 2 min 95℃, 24x (45 s 95℃, 45 s 62℃ - 0.5℃/cycle, 9.5 min 72℃), 20x (45 s 95℃, 45 s 51℃, 4 min 72℃) 10 min 72℃]. The resulting cDNA of the CN129 constructs were cloned in the pCRblunt vector (Invitrogen) for DNA amplification and sub-cloned in the pECFP-N1 vector (Clontech) utilising the restriction sites (5′: NheI, 3′ AgeI) introduced *via* the primers in order to express the constructs as CFP-fusion proteins in eucaryotic cells (see Appendix 6). To obtain negative controls for PKA binding by the introduction of prolines, site directed mutagenesis was performed (QuickChange Site-Directed Mutagenesis Kit, Stratagene, La Jolla, USA) utilising the mutagenesis primers fw-CN129 rat V41P/L45P (5′-GGAGGC TGAGGCCGTTCCCAATGACGTTCCCTTTGCTGTCAACAAC-3′), rv-CN129 rat V41P/L45P (5′-CATGTTGTTGACAGCAAAGGGAACGTCA TTGGGAACGGCCTCAGC-3′), fw-CN129 human V41P/L45P (5′-CGA AGCTGAAGCAGTTCCAAATGATGTTCCCTTTGCTGTTAACAACA TG-3′) and rv-CN129 human V41P/L45P (5′-CAAACATGTTGTTAA CAGCAAAGGGAACATCATTTGGAACTGCTTCAGC-3′) following the instructions of the manufacturer. Inserts of the gained plasmids were controlled by DNA-sequencing.

**Amplification of DNA by polymerase chain reaction**

For the amplification of CN129 cDNA 1.0 $\mu$l Pfu-polymerase (2.5 U/$\mu$l), 0.5 $\mu$l 10x buffer (both Stratagene), 1.0 $\mu$l dNTPs (25 mM), 2.0 $\mu$l $MgSO_4$ (25 mM) 2x 1 $\mu$l oligonucleotides (10 $\mu$M), 1 $\mu$l template (first strand DNA) and 43 $\mu$l *A. bidest* were mixed and reaction performed in the thermocycler with the conditions mentioned above. The resulting DNA was verified by restriction digestion and subsequent analysis on a 1% agarose-gel, and by DNA-sequencing.

### 2.2.2 Immunoblotting

Cell lysates-, cAMP-agarose and immunoprecipitation-samples were boiled (5 min, 95℃) in sample buffer, separated by 8%, 10% or 15% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto polyvinylidene fluoride (PVDF) membranes. The membranes were blocked (Blotto: 5% non-fat dry milk, 0,1% bovine serum albumin (BSA) in TBST, 2 hours at RT or overnight at 4℃) and washed three times for 10 min with TBST and analysed by incubation with specific antibodies. Primary antibodies were applied in Blotto (2 h, RT), membranes were washed (3x 10 min, TBST) and incubated in Blotto (2 h, RT) with a horseradish-peroxidase-conjugated secondary antibody (POD-conjugated Fab-fragments). Membranes were washed (3x 10 min, TBST) and incubated with POD substrate solution (5 min, lumi-light solution, Roche). Signals were visualised with the Lumi-Imager F1 (Roche).

### 2.2.3 Immunoprecipitation

Protein A-conjugated sepharose suspension (30 $\mu$l of 70 mg in 1 ml PBS containing 0.1% azide) was incubated with antibodies (1.5 $\mu$g/$\mu$l if not stated otherwise) and 1 ml of lysate overnight at 4℃. Protein A-conjugated sepharose was washed four times with lysis buffer and eluted with 20 $\mu$l 4x SDS-sample buffer (95℃).

### 2.2.4 Vesicle isolation and PKA activity measurements

AQP2-bearing vesicles were immuno-isolated from rat renal inner-medullary tissue utilising Eupergit C1Z methylacrylate microbeads (Roehm Pharma, Darmstadt, Germany) coated with anti-AQP2 antibodies (AQP2 AB-beads) [71, 111, 72, 112]. Non-saturated binding sites on the beads were blocked with glycine. As a control, beads were coated with glycine alone (control beads). Rat renal inner medullas were homogenised in homogenisation buffer (250 mM sucrose, 3 mM imidazol, pH 7.5). Nuclei and cell debris were removed by sedimentation (3,000 x g, 4℃, 15 min), and the resulting postnuclear supernatants were incubated with AQP2 AB-beads or control beads while rotating (45 min, 4℃). The beads were recovered by sedimentation (3,000 x g, 4℃, 5 min) through a sucrose cushion (0.8 M). The supernatants were discarded and the sediments were washed 5 times by resuspending in PBS and subsequent sedimentation (3,000 x g, 4℃, 5 min). PKA activity was measured using a commercially available assay system based on PKA phosphorylation of the substrate peptide Kemptide (Millipore/Upstate).

### 2.2.5 RII overlay

The RII-overlay method was established by Lohmann *et al.* [113] and was conducted in a modified way as described [114, 98], using $^{32}P$-labelled recombinant human RII$\alpha$, rat RII$\beta$, mouse RII$\alpha$ or a mixture of bovine RII$\alpha$ and RII$\beta$ as stated (mouse RII$\alpha$ subunit overlays were carried out in collaboration with G. McConnachie, member the group of Prof. J.D. Scott, Portland, OR, USA). Briefly, membranes were equilibrated in EtOH (99.9%, RT), washed in PBS and blocked (5% (w/v) non-fat dry milk, 0.1% BSA in PBS with 0.02 % azid) for a minimum of 2 hours. Purified recombinant RII subunits (7,5 $\mu$g) were radiolabelled by incubation with 1 $\mu$l of purified catalytic subunit of PKA (1.66 $\mu$g/$\mu$l, Promega) and 0.1 $\mu$M $[\gamma]^{32}P$-ATP (6000 Ci/mmol; GE Healthcare) in 500 $\mu$l buffer (25 mM $KH_2PO_4$, 10 mM $MgCl_2$, 10 $\mu$M cAMP and 0.5 mM DTT). The final concentration of ATP was reached after 10 min by adding 10 mM cold ATP. After 50 min 70 $\mu$l dextran blue (10 mg/ml) was added and the reaction was stopped by removal of cAMP and the separation of radiolabelled RII subunits using gel filtration (Sephadex G-50, medium; Pharmacia Fine Chemicals). The dextran blue fraction contains the RII subunits. The total activity of the dextran blue fraction was measured and specific activity was calculated in cpm (counts per minute; Liquid scintillation counter Wallac 1410). The membranes were incubated overnight with radiolabelled RII subunits in blocking buffer (specific activity of RII subunits=$(1.4 \pm 0.3) \times 10^8$ c.p.m./$\mu$g of protein per ml of hybridisation solution), washed with blocking buffer (4x, 10 min) and twice with PBS. Signal were detected by autoradiography (Phosphoimager Storm 830) and analysed with the ImageQuant software (GE Healthcare).

### 2.2.6 Peptide synthesis

Peptides derived from the RII-binding domains of AKAPs were synthesised by the Peptide Synthesis group (Dr. Michael Beyermann, FMP) as described [115], for sequences (see table 1.1, 2.1.3 and appendix B) . AKAP7$\delta$-derived peptides were named after the substitution compared with the corresponding position in the full-length AKAP7$\delta$ protein e.g. peptide AKAP7$\delta$-L304T-pep comprises amino acid residues 296-320 of AKAP7$\delta$ with a leucine to threonine substitution at position 304 in AKAP7$\delta$. Peptides with double or multiple substitutions were named accordingly. AKAP7$\delta$-wt (wild-type)-pep comprises the wt sequence of the AKAP7$\delta$ RII-binding domain. For surface plasmon resonance and circular dichroism measurements biotin was attached N-terminally. Peptides were rendered membrane-permeable by N-terminal coupling to stearic acid. The identity of peptides was verified using mass spectrometry (MS). Peptide purities were $> 90\%$ as determined by HPLC analysis (220 nm). Peptide arrays were generated by Angelika Ehrlich (FMP) by automatic SPOT-synthesis on Whatman 50 cellulose membranes by using Fmoc (fluoren-9-ylmethoxycarbonyl) chemistry and the AutoSpot-Robot ASS 222 (Intavis Bioanalytical Instruments AG, Köln, Germany) as described [116, 117, 72]. Control spots (approx. 50 nmol of peptide per spot) were excised from the cellulose membrane and analysed by MALDI-TOF (matrix-assisted laser-desorption ionization-time-of-flight)-MS and HPLC.

### 2.2.7 Cell culture

**HEK293** and **COS7** cells were maintained in DMEM medium supplemented with 10% fetal calf serum, 100 units/ml penicillin G and 100 $\mu$g/ml streptomycin sulfate (all from Invitrogen). Medium for **WT-10** cells was addition-

ally supplemented with 1% non-essential amino acids.

**SH-SY5Y** cells were maintained in DMEM: HAM′s F12 (1:1) medium supplemented with 10% fetal calf serum, 1% non-essential amino acids, 100 units/ml penicillin G and 100 $\mu$g/ml streptomycin sulfate. For confocal laser scanning microscopy, cells were grown on glass cover slips for 48 h. For biochemical analyses, cells were grown for 48-72 h until 80% confluence was achieved.

**Neonatal cardiac myocytes** were preparated as followed: The lower side of the left ventricle of 1-3 day old Wistar rat hearts were isolated, enzymatically digested at 37℃ using 0.48 mg/ml collagenase type II (Biochrom AG, Berlin, Germany) and 0.6 mg/ml pancreatin and suspended in DMEM:M199 (4:1) media supplemented with 10% horse serum (Invitrogen) and 5% foetal calf serum (Invitrogen), and were plated for 1 h on tissue culture plates to deplete fibroblasts. The non-adherent myocytes were plated on 1% (w/v) gelatin pre-coated plates or glass cover slips pre-coated with 0.5 mg/ml Laminin (Roche). After 24 h, the medium was changed to low serum medium (DMEM:M199) containing 4% horse serum.

Primary cultured cells from rat inner medullary collecting duct (**IMCD**) were obtained from 2-3 months old rats as described [118, 99, 119]. Renal inner medullae were excised, dissected in sterile PBS, treated with 0.2% hyalurinidase (Boehringer, Mannheim, Germany) and 0.2% collagenase type CLS-II (Biochrom AG) and incubated for 90 min (37℃, 350 rpm). The cells were washed and resuspended in PBS three times (sedimentation: 1200 x g, 5 min). Cells were subjected to IMCD cell medium (Dulbecco′s modified eagle′s medium (DMEM 5523), 4.5% glucose, 0.5 U/ml penicillin, 0.5 $\mu$g/ml streptomycin, 2 mM glutamine, 1% not essential amino acids, 1% Ultroser,

adjusted to 600 mosmol/l by 100 mM glucose, 100 mM NaCl and 100 mM urea) and seeded on type IV collagen (Becton-Dickinson, Heidelberg, Germany) coated coverslips embedded in 30 $mm^2$ culture dishes to an approximate density of 7.5 x $10^4$ cells/$cm^2$. Media were changed after one and four days of incubation. The culture medium was routinely supplemented with 500 $\mu$M dibutyryl cAMP (dbcAMP; Biolog) for the maintenance of AQP2 expression. Dibutyryl cAMP was removed 16 h prior to experiments, which were performed six days after seeding. Where indicated, cells were incubated with arginine-vasopressin (AVP, 100nM, 15 min).

**Transient transfection of eucaryotic cells**

For transient transfection of HEK293, SH-SY5Y, COS7 or WT-10 cells, Transfectin (Biorad) was combined with serum-free media (DMEM) and DNA according to the instructions of the manufacturer (40 $\mu$l Transfectin/12 $\mu$g DNA per 100 $mm^2$ culture dish) and added to cells grown to 40-60% confluency.

**Preparing lysates from eucaryotic cells**

HEK293 cells were grown in 100 $mm^2$ cell culture dishes for 24-48 h (after transfection) to near confluency. Cells were washed twice with PBS (0℃), and lysis buffer (1.0 ml, 0℃) was added. The cells were then harvested with a rubber policeman, solubilised by vortexing and centrifuged (12,000 x g) for 20 min. at 4℃. The supernatant was collected to final volumes of 1.0 ml if not stated otherwise. Lysates were subjected to Immunoprecipitation or boiled (95℃, 5 min) in appropriate amounts of SDS-sample buffer and analysed in immunoblot or RII overlay experiments.

**Immunofluorescence**

IMCD cells were grown on coverslips. After treatment with AVP and peptidic disruptors (as stated), IMCD cells were washed twice with ice cold PBS, fixed for 15 min (100 mM cacodylate, 100 mM sucrose, 10% paraformaldehyde, 0,2% sodium hydroxide), washed twice (PBS, RT) and permeabilised for 5 min (0.1 Triton X-100 in PBS). After two more washing steps, coverslips were transferred from culture dishes to a coverslip-rack for washing (3x, on shaker 60 rmp) followed by blocking (20 min, 37℃, 1.4‰ fish skin gelatin in PBS). Incubation with the primary antibody (H27, 30 $\mu$l of a 1:300 dilution, 37℃) lasted 45 min followed by washing (3x) and incubation with secondary antibody (Cy3-conjugated goat anti-rabbit antibody, 30 $\mu$l of a 1:300 dilution, 37℃, 45 min). Cells were washed (3x) and embedded by Immunomount (Thermo-Shandon, Pitsburgh, USA).

### 2.2.8 Patch-clamp

Electrophysiological experiments (patch-clamp) were performed by Dr. Dorothea Lorenz (FMP). Cardiac myocytes were obtained from 3-5 day old neonatal rats and cultured as described [120]. The whole-cell L-type $I_{Ca}$ ($Ca^{2+}$ current) was recorded at room temperature from spontaneously contracting cells 3-5 days after seeding [121]. The extracellular solution contained 140 mM TEA chloride, 10 mM Hepes, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 12 mM glucose and 0.1 mM EGTA (pH 7.4 adjusted with CsOH; 300 mOsm/kg). The intracellular solution contained 80 mM CsCl, 15 mM TEA chloride, 20 mM citrate, 10 mM Hepes, 2 mM $CaCl_2$, 3 mM $MgCl_2$, 5 mM EGTA, 10 mM Mg/ATP, 0.3 mM Na-GTP, 0.2 mM free $Mg^{2+}$ and 45 nM free $Ca^{2+}$ (pH 7.4, adjusted with CsOH; 285 mOsm/kg; $Ca^{2+}$ and $Mg^{2+}$ concen-

trations were calculated with Win-MAXC software). Fire-polished recording pipettes (borosilicate glass) were filled with intracellular solution and had a resistance of 2-3 MΩ. To evoke $I_{Ca}$, cells were depolarised repetitively (20 s intervals) from a holding potential of -70 mV to -35 mV with a 400 ms ramp and then depolarised to a test potential of 0 mV for 100 ms. All membrane potentials were corrected for liquid junction potential (11 mV). The currents were filtered at 2.9 kHz and sampled at 2 kHz. If necessary currents were leak-subtracted by the P/4 method. $I_{Ca}$ was measured as the difference between the peak inward current and the current at the end of the test pulse [89]. Series resistance (4-10 MΩ) and total $C_m$ (membrane capacitance) were compensated and continuously recorded. For a comparison of different cells current densities, $I_{Ca}/C_m$ were calculated. Peptides (30 $\mu$M, dissolved in intracellular solution, DMSO content $\leq$ 0.16%) were introduced into the cells via the patch pipette. The influence of peptides on the isoproterenol stimulation of L-type $Ca^{2+}$ channels was measured for 11 min or 22 min (for peptides used at 30 $\mu$M or at 1 $\mu$M, respectively) after patch rupture. Isoproterenol (1 $\mu$M) was applied through an application pipette (QMM Ala Scientific Instruments, New York, USA) positioned near the cell. For statistical analysis (one-way ANOVA) isoproterenol-evoked $I_{Ca}/C_m$ was expressed as a fraction of unstimulated $I_{Ca}/C_m$.

### 2.2.9 Laser Scanning Confocal Microscopy

Laser Scanning Confocal Microscopy (LSCM) was performed with immunostained cells or with transient transfected cells (see above). The fluorophores were excited ($\lambda_{exc}$) and emission ($\lambda_{em}$; filters: BP - band-pass, LP - long-pass) was detected at the following wavelengths DAPI: $\lambda_{exc}$:364 nm,

$\lambda_{em}$:376-451 nm; CFP: $\lambda_{exc}$:458 nm, $\lambda_{em}$:BP 475-525 nm; Cy3: $\lambda_{exc}$:543 nm $\lambda_{em}$:LP 560 nm. The objects were scanned in x/y-direction, the thickness of the confocal z-slices was adjusted to 1 $\mu$m. All confocal images shown in this study show representative images from at least three independent experiments. For immuno-stained cells, negative controls stained with preimmune serum or secondary antibodies alone were routinely carried out. Cells transiently transfected with the CN129-CFP constructs (see above) were compared to mock and untransfected cells.

### 2.2.10 Circular dichroism measurements

Circular dichroism (CD) measurements of N-terminally biotinylated peptides at a concentration of 50 $\mu$M were performed in a mixture of phosphate buffer (10 mM, pH 7.4)/TFE (trifluoroethanol) (1:1, v/v) and, as a control in phosphate buffer (10 mM, pH 7.4) in the presence of 15 mM SDS in a 2 mm cell. Spectra were recorded between 195 and 260 nm on a J-720 spectrometer. The $\alpha$-helicity of the peptides was determined from the $[\theta]_{m.r.w.}$ (mean residue ellipticity) at 222 nm according to the equation $\alpha(\%) = -([\theta]_{m.r.w.} + 2340)x100/30300$ [122].

### 2.2.11 Surface plasmon resonance measurements

Surface plasmon resonance (SPR) measurements were carried out in collaboration by Oliver Diekmann (Biaffin, Kassel, Germany) using a Biacore 2000 instrument (Biacore AB, Uppsala, Sweden) as described [123, 71]. In brief, CM-5 chips (research grade, Biacore AB), coated with streptavidin (200 resonance units, RU; Sigma-Aldrich), were used to capture N-terminally biotinylated AKAP7$\delta$-derived peptides. All subsequent interaction studies were per-

formed in running buffer (20 mM Mops, 150 mM NaCl, pH 7.0 and 0.005% surfactant P20) at 25℃. Non-specific binding was subtracted on the basis of blank surfaces with streptavidin-coated flow cells saturated with either biotin or an appropriate negative control peptide. Regulatory RII$\alpha$ subunits (human; cAMP-free) were injected for 180 s with a flow rate of 50 $\mu$l/min using a series of dilutions (0.5 nM to 1 $\mu$M). After each injection the dissociation phase was monitored for 600 s. Kinetic constants from the raw data were calculated by non-linear regression or equilibrium binding analysis using the Biaevaluation software version 4 (Biacore AB). $K_d$ values were calculated from the respective rate constants on the basis of a Langmuir 1:1 binding model.

## 2.3 Algorithms and bioinformatics

In order to handle the amount of data derived from the database search (see 3.6) computer programs ('perl scripts') were developed utilising the programming language perl including the bioperl modules (for program source codes see Appendix A) . The swissprot identifiers (IDs) used for the retrieval of the cognate protein sequences differ between the scansite database and the swissprot database or changed during the work on this theses. These IDs can be traced with the 'IDtracker' provided on the swissprot homepage.

Searching the scansite database was performed using the regular expression X-X-X-X-X-X-X-X-X-[AVLISE]-X-X-[AVLIF][AVLI]-X-X-[AVLI][AVLIF]-X-X-[AVLISE]-X-X-X-X, where X stand for any and amino acids in square brackets for alternatives, limited to the $P_I$-range of 3.0 to 6.4. All data obtained were stored as plain text files usually in 'comma separated values' (csv)-format which allows further sequence handling with commer-

cially available office programs. The derived peptide-sequences contained sequence combinations that cannot be synthesised in an automatised way [124] or do not fulfil the properties of RII-binding domains (amino acid combinations that are likely to be turn building, proline containing sequences). Such sequences were removed prior to peptide synthesis. In particular sequences serving the following properties were removed: cysteine-containing, five or more sequential alanines, glutamates or leucines and sequences of the pattern X-D-G-X or X-D-S-X (both tend to be turn building). Krchnak *et al.* [124] described peptides that cannot be synthesised by standard methods due to aggregations of certain amino acids and provided a table for the individual amino acids based on empirical studies. This table was utilised to filter this so called 'difficult sequences' by calculating local accumulations of 'problematic' amino acids. A sliding 'sequence window' spanning the length of six amino acids was used to calculate mean values according to the table of Krchnak *et al.* Sequences resulting in mean values lower than 0.4 (corresponding to two or more prolines) or higher than 1.2 (corresponding to 'difficult sequences') were omitted (Fig. 2.1).

The following algorithms are given in pseudo-code.

**Algorithm - sequence retrieval from swissprot database**

```
for all IDs do
    retrieve swissprot database entry
    search sequence for occurring patterns by regular expression
    store ID, matching sequences and Description to file
```

**Algorithm - filter sequence**

```
define "exclude patterns"
define window size
```

```
define value table for amino acids
define lower and upper exclusion value
open "sequence file", store content in "sequence table"

search "sequence table" for "exclude patterns" by regular expression
    store found sequence entries in "excluded file"
    delete found sequence entries in "sequence table"
for all sequences in "sequence table" do
    move window to first position in sequence
    for sequence do
        calculate mean value for window
        if mean value < lower value or mean value > upper value
            store sequence entry in "excluded file"
            delete sequence entry in "sequence table"
        else if
            move window by one position
store remained sequences in "sequence table" to "passed file"
```

Secondary structures of peptides were predicted using the PSIPRED program. Protein and peptide modelling was carried out by Dr. Gerd Krause (Structural bioinformatics and protein design, FMP). For the RII-binding domains of AKAP5 and AKAP13, $\alpha$-helical structures have been determined by NMR [84, 74]. AKAP7$\delta$-derived peptides were docked into the dimerisation/docking domain of RII$\alpha$ subunits (NMR structures of RII$\alpha$ dimers are from the Protein Database (PDB) entries 1R2A and 1L6E that were used as templates) as $\alpha$-helical structures by utilising the tools 'with electrostatic potentials' and 'FlexX' from the SYBYL 6.91 molecular modelling package. The peptide and dimerisation/docking domain complex with fitting shape, complementary electrostatic potentials on the surfaces and the highest aver-

age docking score among the C-Scores (ChemScore, D Score G Score PMF Score) was selected as the best complex. The model of the complex was placed in a water box and minimised with the AMBER 7.0 force field using the conjugated gradient method by 3000 iterations up to the termination gradient of 0.1 kcal/(mol*A) (1 kcal $\approx$ 4.184 kJ). The geometrical quality of the model was validated with PROCHECK.

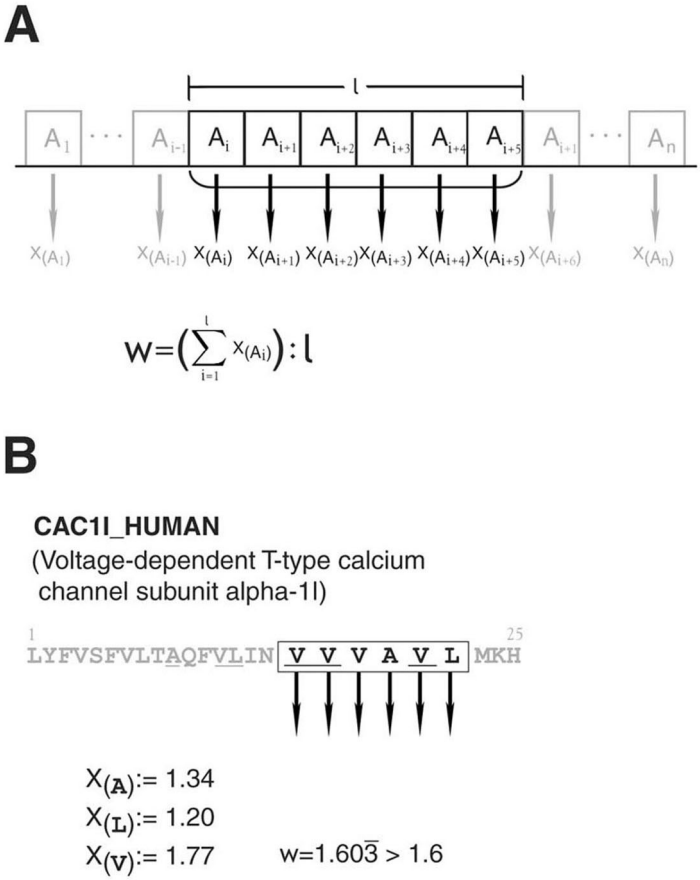


Figure 2.1: Scheme of the filter-algorithm. A) Theoretical scheme of the algorithm to filter so called 'difficult sequences' - an accumulation of unwanted amino acids. A 'sliding window' of length l is utilised to calculate average values for the comprising amino acids. The 'sliding window' moves from position 1 to position n-(l+1) of an amino acid sequence of length n ($A_i$ amino acid in position i ). The average value (w) of the 'sliding window' is calculated by mapping the single amino acids to empiric values $X(A_i)$ according to Krchnak *et al.* (see text). Rising values reflect the rising difficulty to synthesise the corresponding amino acids sequentially. B) Example for a filtered sequence. The sequence of the voltage-dependent T-type calcium channel subunit alpha-1I fits to the search pattern (see text), reflected by the underlined amino acids. Moreover, it is filtered as a 'difficult sequence' as the local accumulation of the depicted amino acids led to an average value $> 1.6$, difficult to synthesise automatically.