2 Materials and methods

All chemicals, if not indicated otherwise, are purchased from Sigma-Aldrich (Munich, Germany) or Roth (Karlsruhe, Germany).

2.1 Biochemistry

2.1.1 Heart homogenate preparation

The lower part of left cardiac ventricle from adult Wistars rat is homogenized in 2-5 ml lyses buffer (20 mM Hepes pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5 % Tween 20) (Asahi et al., 2000) containing 8 µl/ml protease inhibitor stock solution (2 mg/ml soybean trypsin inhibitor, 1.43 mg/ml trasylol (aprotinin), 100 mM benzamidine, 500 µM phenylmethanesulfonyl fluoride (PMSF). For detection of phosphorylated proteins, additionally phosphatase inhibitors are added: 1 mM sodium vanadate and 50 mM sodium fluoride. Samples (1 ml each) are homogenized in a glass/Teflon homogenizer (10 strokes, 1250 rpm) and centrifuged at 24,000 x *g* for 20 min at 4°C. Supernatants are collected for further analyses. In the case of neonatal heart homogenate, 4-6 whole neonatal hearts (not older than 3 days) are homogenized in 2.5 ml lyses buffer as described above.

2.1.2 Cardiac myocyte preparation

Ventricles, isolated from 1-3 day old Wistar rat hearts are enzymatically digested at 37^{o} C using 0.48 mg/ml collagenase type II (Biochrom AG, Berlin, Germany) and 0.6 mg/ml pancreatin (Sigma-Aldrich, Munich, Germany) and suspended in DMEM: M199 (4:1) media supplemented with 10 % horse serum (Invitrogen-Gibco, Karlsruhe, Germany) and 5 % foetal calf serum (Invitrogen-Gibco, Karlsruhe, Germany). Fibroblasts are depleted for 1 h on tissue culture plates. The non-adherent myocytes are plated on 1 % (w/v) gelatine coated plates or glass cover slips coated with 0.5 mg/ml Laminin (Roche, Mannheim, Germany). After 24 h, the medium is changed to low serum medium (DMEM:

M199) containing 4 % horse serum. Myocytes are used for experiments from day 3-5 of cultivation.

2.1.3 Cell lysis

For preparation of cell lysate, the cultured neonatal cardiac myocytes or HEK293 cells are scraped from the plates with lysis buffer (10 mM K_2HPO_4, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 1 % Triton X-100 and protease inhibitor mix 0.2 % deoxycholate, 3.2 µg/ml trypsin inhibitor I-S, 1.4 µg/ml aprotinin, 1 mM benzamidine and 0.5 mM phenylmethanesulfonyl fluoride). The lysates are cleared by centrifugation (12,000 x *g*, 4 °C and 30 min).

2.1.4 RII-overlay

RII-overlays are conducted as described in (Bregman et al., 1989; Klussmann et al., 1999) using ^{32}P-labeled recombinant human RIIα, which was kindly provided by the University of Kassel, Fritz Herberg. Briefly, the PVDF membrane containing the samples is blocked in blotto (5 % [w/v] non-fat dry milk plus 0.1 % bovine serum albumin (BSA) in Phosphate-buffered saline (PBS) with 0.02 % Na-azide) for a minimum of 2 hours. RII subunits (7.5 µg) are radiolabeled with 1 µg of purified catalytic subunit (C) of PKA (0.02 µg/µl, Promega, Madison, USA) and 0.1 µM [γ-^{32}P] ATP (75 µCi) (Amersham Biosciences, Freiburg, Germany), 25 mM potassium phosphate, 10 µM cAMP, 10 mM $MgCl_2$, and 0.5 mM DTT in 500 µl total volume. After 10 min 10 mM cold ATP is added. After 50 min dextran blue is added and the reaction mixture is fragmentized using gel filtration (G-50 sepharose). The dextran blue fraction contains the radiolabeled RII subunits. The PVDF membrane containing the samples is incubated over night with 10^5 cpm/ml radiolabeled RII subunits in blocking buffer. Membranes are washed four times with blocking buffer and twice with PBS. Radiolabeled RII subunits are detected by the Phosphoimager Storm 830 system (Amersham Biosciences, Freiburg, Germany). Preincubation for 30 minutes before incubating with the radiolabeled RII subunits with 500 nM of the synthetic peptide Ht31, or with 10 nM of the peptide L314E, preventing the binding between AKAPs and the regulatory subunits of PKA proves, that the

signals disappearing after peptide treatment are specific for AKAPs. Ht31 forms an amphipatic helix, which can bind PKA RII subunits with a nanomolar affinity, and is therefore competitively inhibiting the AKAP-PKA-RII binding (Zakhary et al., 2000a). L314E is a peptide derived from the RII binding site of AKAP18δ, competitively displacing all other AKAPs from binding to PKA RII subunits (Hundsrucker et al., 2006).

2.1.5 Western blot (WB)

Cell lysates or immunocomplexes, are analyzed on a 10% or 15% SDS/PAGE and blotted onto polyvinylidene difluoride (PVDF) membranes. The filters are blocked in 5% non-fat dry milk in Tris-buffered saline Tween-20 (TBST), (0.1 mM Tris, 1.5 mM NaCl, 0.05 % Tween20, pH 7.4) for 1 hour at room temperature, incubated 2 hours at room temperature with primary antibodies, (see table 2) washed three times for 10 min in TBST and incubated with a horseradish-peroxidase-conjugated secondary antibody (Dianova Hamburg, Germany). Signals were visualized with the Lumi-Imager F1 (Roche Diagnostics, Mannheim, Germany) using lumi-light solution according manufacturers instructions (Roche Diagnostics, Mannheim, Germany).

2.1.5.1 Antibodies

Name	Recognized protein	Epitope/ Antigene	Isotope	Company	one IP	Dilution WB	Dilution IF	Reference
A18δ4	AKAP18δ	Amino acids 1-33 from AKAP18δ	Rabbit, polyclonal	BioGenes, Berlin, Germany	10-30µl	1/500	1/100	(Henn et al., 2004)
A18δ3	AKAP18γ,δ	Amino acids 60-76 from AKAP18δ	Rabbit, polyclonal	BioGenes, Berlin, Germany	10-30µl	1/250	1/100	
1964	AKAP18α,β,γ,δ	Amino acids 341-353 from AKAP18δ	Rabbit, polyclonal	BioGenes, Berlin, Germany	30µl	1/250	1/100	-
A18δ5	AKAP18α,β,γ,δ	Amino acids 1-353 from AKAP18δ	Rabbit, polyclonal	BioGenes, Berlin, Germany	15µl	1/500		-
GFP02	GFP,YFP, CFP,GST	GFP-GST fusion protein	Rabbit, polyclonal	BioGenes, Berlin, Germany	1-5µl			(Alken et al., 2005)
GFP	GFP,YFP, CFP		Mouse, mono-clonal	Clontech Laborator-ies, Mountain View, USA	Not teste d	1/5000	-	

Name	Recognized protein	Epitope/ Antigene	Isotope	Company	one IP	Dilution WB	Dilution IF	Reference
PLB 2D12	PLB	Amino acids 2-25 from PLB	Mouse, mono-clonal	Affinity Bio Reagents	0.5-2µl	1/5000	1/100	(Huke and Periasamy, 2004)
phospho-PLB (Ser16)	Ser16 phospho-PLB	Amino acids 14-25 from PLB	Rabbit, polyclonal	Upstate Biotechno-logy Lake Placid, NY, USA	Not tested	1/2500	Not tested	(Suresh Babu et al., 2007)
SERCA 2a 2-7-A1	SERCA2a	Full length canine SERCA2a	Mouse, mono-clonal	Abcam, Cambridge, UK	-	1/500	1/100	(Temsah et al., 1999)
SERCA 2a C-20	SERCA2a	C-terminus human	Goat, polyclonal	Santa Cruz Biotechno-logy, Santa Cruz, Ca, USA	1/250	1/100	1/100	(Behne et al., 2003)
α-actinin EA-53	sarcomeric α-actinin	Full length rabbit protein	Mouse, mono-clonal	Sigma-Aldrich, Saint Louis, Missouri, USA	Not tested	1/2000	1/1000	(Goncharova et al., 1992)
PKA RI	PKA RI	amino acids 225-381 of mouse PKA RI	Mouse, mono-clonal IgG2b	BD Trans-duction Labora-tories, Lexington, KY, USA	yes	1/250	1/50	(Tasken and Aandahl, 2004)
PKA RIIα	PKA RIIα	Human PKARIIα 18-347	Mouse, IgG1 Mono-clonal	BD Trans-duction Labora-tories, Lexington, KY, USA	-	1/1000	1/50	(Tanji et al., 2002)
PKA RIIβ	PKA RIIβ	Human PKARIIβ 18-347	Mouse, IgG1 Mono-clonal	BD Trans-duction Labora-tories, Lexington, KY, USA	yes	1/1000	1/50	(Budillon et al., 1995)
PKA C	PKA C	Human PKACα 18-347	Mouse, mono-clonal IgG2b	BD Trans-duction Laboratorie s, Lexington, KY, USA		1/500		(Westphal et al., 1999)
Calsequestrin	Calseques-trin	Peptide of amino residues 39-48 of cardiac calseques-trin	Rabbit, polyclonal	Upstate Biotech-nology Inc.,Lake Placid,NY, USA	0,5 µg/µl	1/2500	1/125	(MacLennan, 1970)
AQP2	AQP2		Rabbit, polyclonal	BioGenes, Berlin, Germany		1/2500	1/600	(Liebenhoff and Rosenthal, 1995)
PDE4D 3/9	PDE4D3/9		Sheep polyclonal	SAP, ∗4	0.5µl	1/5000	1/500	(Shepherd et al., 2004)

Name	Recognized protein	Epitope/ Antigene	Isotope	Company	one IP	Dilution WB	Dilution IF	Reference
POD	Mouse/ rabbit	IgGs		Jackson Immuno-research, Newmarket, UK	-	1/400	-	
Cy3/Cy5	Mouse/ rabbit	IgGs		Jackson Immuno-research, Newmarket, UK	-	-	1/600	

Table 2 Description of the different antibodies used in this work

2.1.6 Immunoprecipitation

Heart homogenate or cell lysate is incubated over night at 4° C with the correspondent antibody (described in table 2) and 2 mg protein A-conjugated agarose (Sigma Deisenhofen, Germany). Immunocomplexes are washed four times in the respective lysis buffer, boiled in SDS Laemmli sample buffer, resolved by SDS/PAGE and detected by immunoblotting.

2.1.7 cAMP pull down

For cAMP-agarose pull-down experiments the homogenates are incubated with cAMP-agarose (Biolog, Bremen, Germany) during 3 h at 4° C in the absence or presence of an excess of cAMP (50 mM). Proteins bound to cAMP conjugated agarose are washed four times with lyses buffer and eluted with Laemmli sample buffer (95° C), separated by SDS/PAGE and detected by immunoblotting.

2.1.8 Immunofluorescence microscopy

Rats (*R. norvegicus*) are sacrificed. The left ventricles of the hearts are removed and cut into smaller fragments using a razor blade. The tissue is shock frozen in liquid nitrogen, embedded in Tissue Embedding Medium (Jung, Tissue freezing medium, Leica Instruments GmbH, Nussloch, Germany) mounted in the cryostat (Cryostat CM 3000 from Lyeka) and cut into sections of 5-10 µm at a temperature of -20° C. Sections are transferred from the knife to a glass slide (Menzel, Superfrost Plus, Germany) and fixed with 2.5 % paraformaldehyde in sodium cacodylate buffer (100 mM sodium cacodylate and 100 mM sucrose, pH 7.4) for 30 min. The slides are washed three times with phosphate-buffered

saline (PBS). Cells are permeabilized in PBS containing 0.1 % Triton X-100 for 5 min and washed three times. Blocking is carried out in blocking solution (0.3 ml 45 % fish skin gelatine/100 ml PBS) in a humidifying chamber by incubation for 45 min at 37° C. After three washes with PBS (10 min each wash), antibody diluted in blocking solution is added. The slides are incubated in a humidifying chamber for 45 min at 37° C, and washed again three times (10 min each wash) with cold PBS, and subsequently incubated in a humidifying chamber for 45 min at 37° C with Cy3 or Cy5 labeled secondary antibody. Cover slips are mounted with Immu-Mount (Thermo Shandon, Thermo Electron Corporation, Dreieich, Germany) according to the manufactures instructions. Samples are visualized by confocal microscopy using a Zeiss laser scanning microscope LSM 510 (Zeiss, Jena, Germany).

2.1.9 Immunogold electron microscopy (EM)

Immunogold electron microscopy (EM) is performed by Dorothea Lorenz and R. Ringling, FMP-Berlin. Immunogold electron microscopy is carried out as described (Henn et al., 2004). Hearts are obtained from neonatal rats, fixed (0.25 % glutaraldehyde, 3 % formaldehyde), cryosubstituted in a Leica AFS freeze-substitution unit and embedded in LR-White. The samples are sequentially equilibrated over 4 days in methanol at temperatures gradually increasing from -90° C to -45° C. The samples are infiltrated with LR-White for 72 h at -20° C and polymerized for 1 h at -20° C and 2 h at 4° C. Sections (60 nm) are cut on a Reichert Ultracut S, placed on nickel grids and blocked with glycine. The sections are incubated with mouse anti-PLB (Affinity Bioreagents, Golden, Co, USA) and goat anti-SERCA2 antibody (Affinity Bioreagents, Golden, Co, USA). The sections are washed with PBS and incubated with anti-mouse antibody and anti-goat antibody coupled to 15 and 10 nm gold grains, respectively. Co-staining of PLB and AKAP18δ is performed with primary mouse anti-PLB and affinity-purified rabbit anti-AKAP18δ (Henn et al., 2004) antibodies. For co-staining of SERCA2 and AKAP18δ, mouse anti-SERCA2 (Affinity Bioreagents, Golden, Co, USA) and affinity-purified rabbit anti-AKAP18δ A18δ_4 antibody are used. All primary antibodies are diluted 1:100. All secondary antibodies (Jackson Immunosearch, Newmarket, UK) are applied in 1:20 dilutions. The sections are stained with uranyl acetate and lead citrate

before analysis with an 80 kV electron microscope (902A, LEO, Obercochem, Germany) equipped with a slow scan CCD camera (Megaview III, Soft Imaging System, Germany) and the analySIS software.

2.2 Plasmids and fusion proteins

2.2.1 Polymerase chain reaction (PCR)

All primers (25-30 bases in length) are purchased from Biotez, Berlin Buch, Germany and diluted to 20 nM in water for further use.

To later on clone PLB, AKAP18δ or PDE4D DNA into the corresponding expression vectors, DNA is amplified by PCR adding the restriction sites (see 2.11.3-5). Template DNA is diluted to 50 µl reaction mixture as described below:

5 µl 10x Pfu-buffer (Stratagene, Cedar Creek, Texas, USA)
1 µl dNTPs (20nM) (Fermentas, St-Leon-Rot, Germany)
2 µl $MgSO_4$ (Stratagene, Cedar Creek, Texas, USA)
1 µl Pfu (Stratagene, Cedar Creek, Texas, USA)
1 µl forward primer (20nM)
1 µl reversed primer (20nM)
20 ng template DNA
ad 50 µl H_2O

PCR is performed in a Thermocycler (GeneAmp PCR system 9700, Applied Biosystems, Foster City, USA) with the following program:

1.) 94°C 2 min
2.) 94°C 30 s
3.) 58°C 40 s
4.) 72°C 1 min/kb DNA template
5.) 72°C 7 min
6.) 4°C ∞
cycle 2.) to 4.) are repeated 30 times

2.2.2 Mutagenesis

All primers (25-30 bases in length) are purchased from Biotez, Berlin Buch, Germany and diluted to 20 nM in water for further use. All restriction enzymes are purchased from New England Biolabs, Ipswich, MA, USA. Mutagenesis forward and reverse primers contain the mutated sequence in the middle, flanked by the original sequence of the template DNA. Template DNA is diluted to 50 µl reaction mixture as described below:

2.5 µl 10x buffer B (Roboklon, Berlin-Buch, Germany)
1 µl dNTPs (20nM) (Fermentas, St-Leon-Rot, Germany)
0.75 µl Pfu (Roboklon, Berlin-Buch, Germany)
1 µl forward primer (20 nM)
1 µl reversed primer (20 nM)
20 ng template DNA
ad 25 µl H_2O

Mutagenesis is performed in a Thermocycler with the following program:

1.) 94°C 2 min
2.) 94°C 30 s
3.) 58°C 40 s
4.) 72°C 1 min/kb DNA template
5.) 72°C 7 min
6.) 4°C ∞
cycle 2.) to 4.) are repeated 30 times

PCR products are digested with 1 µl DnpI/sample for 1-3 h, and than transformed in electro-competent E.coli Top10 cells. All constructs are sequenced prior to expression.

2.2.3 AKAP18δ constructs

AKAP18δ-YFP	pEYFP-N1
AKAP18δ-CFP	pECFP-N1
AKAP18δ-YFP	pEYFP-C1
AKAP18δ-N67	pEYFP-C1
AKAP18δ-N124	pEYFP-C1
AKAP18δ-N201	pEYFP-C1
AKAP18δ-N301	pEYFP-C1
AKAP18δ-C301	pEYFP-C1
AKAP18δ-R66E	pEYFP-N1
AKAP18δ R116A/R120A	pEYFP-N1
AKAP18δ-GST	pEGEX-4T3
AKAP18δ-	pEYFP-N1

AKAP18δ is ligated into the vector pGEX-4T-3 (Amersham Biosciences, Freiburg, Germany), as described in (Henn et al., 2004). For generating a plasmid encoding a fusion protein of AKAP18δ with the cyan fluorescent protein (CFP) or the yellow fluorescent protein (YFP), full-length AKAP18δ is amplified by PCR using GST-AKAP18δ (see above) as template and forward and reverse primers containing EcoRI and BamHI restriction sites, respectively. The digested PCR product is sub-cloned into the vector pECFP-N1 (BD Biosciences), (Henn et al., 2004). For construction of truncated AKAP18δ-YFP, AKAP18δ-YFP is used as template. Forward primers containing an EcoRI restriction site and a start codon followed by a sequence starting at bp 201, 372, 603 or 903 and reverse primers containing a stop codon and BamHI restriction site are used for the amplification. The resulting PCR products are ligated into vector pEYFP-C1 (BD Biosciences, Heidelberg, Germany). For the construct AKAP18δ-C301, a forward primer containing an EcoRI restriction site with start codon is combined with a reversed primer coding for a stop codon after bp 1059 for. Additionally, AKAP18δ is cloned in a fusion vector between pEYFP-N1 and pEYFP-C1 allowing expression of protein without GFP tag. In order to substitute the arginine in position 66 to glutamate (R66E), or the arginine in position 116 and 120 to alanine (R116A/R120A) of AKAP18δ-YFP, site-directed

mutagenesis is performed as described above. All constructs are sequenced prior to expression.

2.2.4 PLB constructs

PLB-wt	pEYFP-C1
PLB-N17	pEYFP-C1
PLB-N26	pEYFP-C1
PLB-C30	pEYFP-C1
PLB-L31A	pEYFP-C1
PLB-L37F	pEYFP-C1
PLB-RR13/14PP	pEYFP-C1
PLB-R25P	pEYFP-C1
PLB-S16E	pEYFP-C1
PLB-S16D	pEYFP-C1

For generating a plasmid encoding a fusion protein of human PLB with CFP or YFP, full-length human PLB is amplified by PCR using as template human PLB, NM_002667 in pDualGC vector, purchased from the expression-tested human clone collection (Stratagene, Cedar Creek, Texas, USA). Using forward and reverse primers containing EcoRI and BamHI restriction sites respectively, PLB is subcloned into the vector pECFP-C1 (BD Biosciences, Heidelberg, Germany). For cloning of PLB-YFP deletion mutants, PLB -YFP is used as template for PCR. Forward primers containing an EcoRI restriction site and a start codon followed by the sequence starting at bp 50 or 81 respectively, and reverse primers containing a stop codon and BamHI restriction site are used for amplification. The resulting PCR products were cloned into pEYFP-C1. For the construct PLB-C30, base pairs 91-93 are mutated to the stop codon tga. Mutagenesis for the PLB constructs is performed as described above.

2.2.5 PDE4D3 constructs

Constructs of human PDE4D3 cDNA with a C-terminal VSV epitope tag in the plasmid pcDNA3 (Invitrogen, Paisley, Scotland, UK), and a mutated construct where amino acids FQF 598-600 of the ERK binding site are changed to alanin are a friendly gift from the group of Miles Houslay, University of Glasgow. We subcloned full lengths PDE4D3 into pGEX4T3 and pEGFP-N1 using primers with EcoRI and BamHI restriction sites and PDE4D3-vsv as a template for PCR amplification. PCR primers adding an N-terminal flag tag and EcoRI and BamHI restriction sites to the construct are used to clone the PDE4D3 constructs PDE4D3-flag, PDE4D3-flag-N390, and PDE4D3-flag-C560. PCR fragments contain a stop codon at the end, and are ligated into vector pEYFP-N1, expressing only the flag-, and no YFP-tag.

2.2.6 Transfection of HEK293 cells.

HEK293 cells (ATTC, USA) at 40-50 % confluence are transfected with 1 µg of plasmid DNA per 9,6 cm^2 culture dishes using 1.5 µl TransFectin™ Lipid Reagent according to the manufactures instructions (BIO-RAD, USA). Cells are lysed 24 h after transfection.

2.3 Experiments performed at the University of Oslo

2.3.1 Heart sub-cellular fractionation

Rat hearts are fractionated according to a modified protocol described in (Kapiloff et al., 2001). In brief, two rat hearts (Pel-Freeze Biologicals, Rogers, AR, USA) are homogenized using a mortar in 20 ml buffer B (10 mM Hepes pH 7.4, 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), 1 mM benzamidine, 5 mM EDTA, protease inhibitors (Complete Mini, EDTA-free tablets, Roche)) with 0.32 M sucrose. Whole heart homogenate is filtered through cheesecloth, and centrifuged at 3,800 x *g* for 20 min. The supernatant (S1) is centrifuged again at 100,000 x *g* for 1 h. The resulting pellet (P2), containing SR, Golgi apparatus and plasma membrane, is resuspended in 2 ml buffer B with 0.32 M sucrose. Purified SR is obtained by centrifugation of P2

(layered on top of 8 parts 24 %, 6 parts 40 %, 2 parts 50 % sucrose in 5 mM Hepes buffer) at 100,000 x *g* for 90 min through a sucrose gradient. SR forms a layer at the interface between 24 % and 40 % sucrose recovered in fractions 9-13 from the top. All centrifugation steps are carried out at 4^{o} C. Fractions are separated by SDS/PAGE and proteins detected by immunoblotting or RII-overlay as described above.

2.3.2 Peptide spot experiments

Peptide arrays are synthesized on cellulose paper by using Multipep automated peptide synthesizer (INTAVIS Bioanalytical Instruments AG, Germany) as described (Frank, 1992; Hundsrucker et al., 2006; Stefan et al., 2007). Interaction of spotted peptides with GST-AKAP18δ or GST is tested by overlaying the membranes with 1 µg/ml of recombinant protein in TBST. Bound recombinant protein is detected with anti-GST antibody. The procedure and detection of signals is identical with immunoblot analysis (see above).

2.3.3 Protein expression and purification

GST-AKAP18δ or GST alone is expressed in *E.coli* BL21 after IPTG induction. Cells are centrifuged and the GST-AKAP18δ or GST containing pellet is incubated in lysis buffer (10 mM MOPS pH 6.5, 100 mM NaCl, protease inhibitors (Complete Mini, EDTA-free tablets, Roche)) and sonicated (UP400s ultrasonic processor) three times for 1 min at 0°C. Homogenized cells are centrifuged and the supernatant is incubated with glutathione-agarose beads (Sigma) and rotated overnight at 4°C. The recombinant protein bound to the beads is washed by low speed centrifugation twice in lysis buffer and twice in washing buffer (5 mM MOPS pH 6.5, 0.5 M NaCl) and again twice in lysis buffer. The recombinant protein is eluted with 20 mM L-Glutathione (reduced, in 50 mM Tris-HCl pH 8.4 150 mM NaCl) at 4 °C overnight and subsequently dialysed against PBS overnight.

2.3.4 siRNA

siRNA sequences used are synthesized at Oslo University or by Dharmacon. Transfection of siRNA against AKAP18δ and protein expression knock-down is tested in HaCaT cells before examining effects in neonatal cardiac myocytes. Tests are made with three different siRNAs for AKAP18δ, to make sure that the observations made are not off-target effects. In order to identify neonatal cardiac myocytes transfected with siRNA, siRNA is labeled with Cy3. The sequences of the siRNAs shown are:

AKAP18δ: 5' GGG AGA AAU AGA UGC CAA UAA 3'
5' AUU GGC AUC UAU UUC UCC CGC 3'

Control: 5' GGG ACA AAU ACA UGG CAA UAA 3'
5' A UUG CCA UGU AUU UGU CCC GC 3'

2.4 Experiments performed at the University of Padua

2.4.1 Ca^{2+}imaging

Neonatal cardiac myocytes are transfected (TransFectin Lipid Reagent, Bio-Rad) with the FRET-based Ca^{2+} sensor Chameleon D1ER, see Fig. 2.1, (Miyawaki et al., 1997) targeted to the SR in the absence or in the presence of 50 nM of previous described siRNA. Real-time imaging experiments are performed after 48 h transfection in a modified Ringer's solution containing 1 mM glucose, 30 mM KCl and either 300 µM EGTA or 3 mM Ca^{2+}. Cells are imaged (1 frame/10 s) in the presence of 50 µM of the SERCA2 inhibitor 2,5-Di-*tert*-butylhydroquinone (BHQ) (Sigma-Aldrich) and in the presence of 300 µM EGTA to monitor the release of Ca^{2+} from the SR. BHQ was subsequently washed away with a solution containing 1 mM glucose, 30 mM KCl and 300 µM EGTA. Subsequent Ca^{2+} re-uptake into the SR is monitored by Ca^{2+} imaging (1 frame/250 ms) in the presence of 3 mM Ca^{2+}. In cells treated with 10 µM of the ß-AR agonist norepinephrine (NE), NE is added at the start of the washing out phase and for the entire duration of the experiment. The value of the time

constant τ is calculated by fitting the recovery phase in the curve of Ca^{2+} re-uptake by using the exponential function $f(t) = \sum^{n}_{i=1} A_i e^{-t/\tau i} + C$.

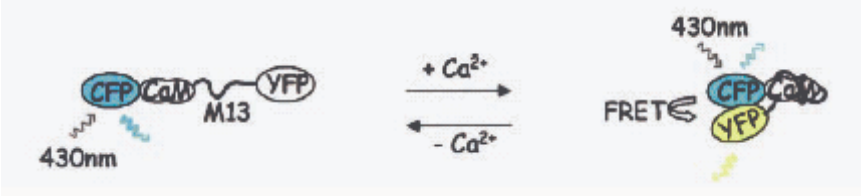

Figure 2.1 The tandem fusion protein of CFP, YFP and CaM, (CaM-binding domain from smooth muscle myosin light chain kinase M13) forms a sensor for intracellular free Ca^{2+}. Arrows indicate CFP excitation (430 nm), CFP emission (480 nm) and YFP emission (545 nm).

2.5 Renal inner medullary collecting duct (IMCD) cells

2.5.1 Culture of primary rat inner medullary collecting duct (IMCD) cells

Primary cultures of IMCD cells are obtained from 2-3 month old Wistar rats and prepared as described in (Maric et al., 1998b). Briefly, rats are sacrified by decapitation, and kidney inner medulla, including papilla, is removed and cut into small pieces. Tissue is digested in PBS containing 0.2 % hyaluronidase (Boehringer, Mannheim, Germany) and 0.2 % collagenase type CLS-II (Biochrom, Berlin, Germany) at 37° C for 90 min. The cells are washed 3 times with PBS by centrifugation for 5 min at 500 x g. Cells are seeded at a density of 7,5 x 10^4 cells/cm^2 on petri dishes or glass coverslips coated with type IV collagen (5 µg/cm^2) (Becton-Dickinson, Heidelberg, Germany). Per sacrified animal, 20–22 cm^2 of confluent IMCD cells are yielded resulting in approximately 1,25 culture dishes of 35 mm diameter. Cells are cultured in DMEM 5523, supplemented with 1 % non-essential amino acids, 0.5 µg/ml streptomycin and 1 % of ultroser (Life Technologies, Karlsruhe, Germany). Because IMCD cells are adapted to high osmotic challenges within the kidney medulla, the medium is adjusted to 600 mosmol/l by the addition of 100 mM glucose, 100 mM NaCl and 100 mM urea to establish growth conditions with preferential selectivity for IMCD principal and intercalated cells (Mooren and Kinne, 1994), 500 µM dibutyryl cAMP (dbcAMP; Biolog, Bremen, Germany) are

added during the culture for the maintenance of AQP2 expression. Cells are incubated at 37° C and 5 % CO_2. Medium is changed after 1 and 4 days, and cells are used after 5-7 days. Dibutyryl cAMP is removed 16 h prior to experiments.

2.5.2 Substances for treatment of IMCD cells

5, 6- Dichloro-1- ß- D- ribofuranosylbenzimidazole- 3', 5'- cyclic monophosphorothioate, Sp- isomer (Sp-5,6-DCl-cBIMPS),

N^6- Mono- tert.-butylcarbamoyladenosine- 3', 5'- cyclic monophosphate (6-MBC-cAMP), N^6,

8-Bromoadenosine-3', 5'-cyclic monophosphorothioate, Rp isomer (Rp-8-Br-cAMPS)

2'- O- Dibutyryladenosine- 3', 5'- cyclic monophosphate (dbcAMP),

Dibutyryl cAMP (dbcAMP) and all other cAMP analogues are generously gift from Biolog (Bremen, Germany).

Forskolin, Sigma-Aldrich (Munich, Germany

Arginin vaopressin (AVP), synthesized by Michael Beyermann, FMP-Berlin

IMCD cells are incubated for 15 minutes with cAMP analogues. For preferential stimulation of RII subunits, a combination of Sp-5,6-DCl-cBIMPS and 6-MBC-cAMP at a concentration of 25 µM is used. This combination shows a selectivity of 1:60 for PKA I/PKA II (Schwede et al., 2000). To further increase the selectivity, the PKA inhibitor Rp-8-Br-cAMPS is added. This analogue shows an inhibitory effect with a selectivity of 1/23 for PKA I/ PKA II.

2.5.3. Detection of AQP2, RI and RII subunits of PKA in IMCD cells by immunofluorescence microscopy

Laser scanning microscopy is carried out as described above (paragraph 2.1.9), antibody concentrations, see table 1. To quantify the effects of AVP and cAMP analogues on the localization of AQP2 and R subunits, the intracellular/plasma membrane fluorescence signal intensities are determined (Maric et al., 1998a; Tamma et al., 2003; Henn et al., 2004; Klussmann et al., 1999; Klussmann and

Rosenthal, 2001). In addition, for approximately 100 IMCD cells, the percentage of cells presenting AQP2 in the plasma membrane is determined.

2.5.4 Immunoisolation of intracellular vesicles and preparation of sub-cellular fractions

AQP2-bearing vesicles are immunoisolated as described in (Nedvetsky et al., 2006; Stefan et al., 2007; Hundsrucker et al., 2006; Henn et al., 2004). In brief, affinity-purified AQP2 antibodies are coupled to Eupergit C1Z methacrylate microbeads (Roehm Pharma, Darmstadt, Germany) according to the procedure described (Burger et al., 1989), yielding AQP2AB beads. Non-saturated binding sites are blocked with glycine. As a control, beads are coated with glycine alone (control beads). Rat renal inner medullae are homogenized in homogenization buffer containing 250 mM sucrose, 3 mM imidazol, pH 7,5. Nuclei and cell debris are removed by centrifugation (3,000x*g*, 4 °C, 15 min), and the resulting post-nuclear supernatants are incubated with AQP2AB or control beads while rotating (45 min, 4 °C). The beads are recovered by centrifugation (3,000 x *g*, 4 °C, 5 min) through a sucrose cushion (0.8 M). The supernatants are discarded and the pellets are washed by 5 rounds of resuspending in PBS and subsequent centrifugation (3,000x*g*, 4 °C, 5 min). The final pellets are resuspended in Laemmli sample buffer and subjected to Western blotting (see below). For immuno-isolation of intracellular vesicles 6 dishes (60 mm) of IMCD cells (6 x 10^6 cells/dish) are used.

To obtain particulate and soluble fractions from 60 mm dishes of IMCD cells are homogenized in standard lysis buffer and cleared from cell debris and nuclei (4,000x*g*, 4 °C, 10 min), and centrifuged (150,000x*g*, 4 °C, 1 h). The supernatant and the pellet, resuspended in lysis buffer, are considered soluble and particulate fractions, respectively (Henn et al., 2004; Klussmann and Rosenthal, 2001). Proteins are analyzed by Western blotting (see below).