3 Materials

3.1 Reagents and substances

Reagents	Purchased
2-mercaptoethanol	Carl Roth, GER
8-arginine vasopressin	Leibniz-Institut für Molekulare Pharmakologie (FMP), GER
Acetic acid	VWR Prolabo, F
Acetonitrile	Carl Roth, GER
Adenosine triphosphate	Sigma, USA
Agar	Life Technologies, GER
Agarose	Sigma, USA
Albumin standard 2.0 mg/ml	Pierce, USA
Ammonium bicarbonate	Merck, GER
Ammonium chloride	FLUKA Chemie, CH
Ammonium persulfate (APS)	Merck, GER
Ammonium sulfate	Merck, GER
Aprotinine	Merck, GER
Aquasafe 300 plus	Zinsser Analytik, GER
Benzamidine	AppliChem, USA
Bovine serum albumine (BSA)	Sigma, USA
Bromphenol blue	Carl Roth, GER
Cacodylic acid sodium salt	Carl Roth, GER
Calcium chloride	Merck, GER
Chemiluminescence detection kit for POD	AppliChem, USA
Chloroquine diphosphate salt	Sigma, USA
Coomassie G250 brilliant blue	Serva, GER
Creatine phosphokinase	Sigma, GER

Cyclic adenosine 3', 5'-monophosphate	Sigma, GER
Cycloheximide	Calbiochem, USA
D-Glucose	Carl Roth, GER
D-(+)-Sucrose	Carl Roth, GER
Dimethyl sulfoxide (DMSO)	Sigma, GER
Disodium hydrogenphosphate	Merck, GER
Dried milk powder (low fat)	AppliChem, USA
Ethanol	J.T. Baker, NL
Ethidium bromide	Carl Roth, GER
Ethylenediaminetetraacetic acid (EDTA)	Sigma, USA
Forskolin	Sigma, USA
Glycerine	AppliChem, USA
Glycine	AppliChem, USA
Guanosine triphosphate (GTP)	Sigma, GER
Half Dye	Bioline, USA
HCl	J.T. Backer, NL
HEPES (4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid)	Carl Roth, GER
IBMX (3-Isobutyl-1-methyl-Xanthin)	Sigma, GER
Imidazole	Sigma, GER
Immu-Mount™ mounting medium	Thermo Shandon, USA
Isopropanol	Merck, GER
Lactacystin	Sigma, GER
L-Cysteine	Sigma, GER
LipofectAMINE™ 2000 reagent	Invitrogen, USA
L-Methionine	Sigma, GER
LysoTracker® Red	Invitrogen, USA
Magnesium chloride	Merck, GER
Magnesium sulfate	Merck, GER
Mangan chloride	Merck, GER

Methanol	J. T. Backer, NL
MG132, proteasome inhibitor	Calbiochem, GER
N-ethylmaleinimide	AppliChem, USA
Nitrocellulose (Optitran BA-S 85)	Schleicher & Schuell, GER
N,N,N`,N`,- tetramethylethylene diamine	Sigma, USA
Orthophosphoric acid	Merck, GER
Paraformaldehyde	Merck, GER
Pfu reaction buffer 10 x	Stratagene, USA
Phenylmethylsulfonyl fluoride (PMSF)	Carl Roth, GER
Phospho-creatinine	Sigma, GER
PIPES (piperazine-1,4-bis(2-ethanesulfonic acid)	Sigma, GER
Poly-L-Lysine	Sigma, USA
Ponceau S	Roche, CH
Potassium chloride	Merck, GER
Potassium dihydrogenphosphate	Merck, GER
Protein A-Sepharose	Sigma, USA
Roti-Load 1 (protein loading buffer, reducing)	Carl Roth, GER
Rotiphorese-gel 30 (37.5:1)	Carl Roth, GER
Sodium chloride	AppliChem, USA
Sodium deoxycholate	Serva, GER
Sodium dihydrogenphosphate	Merck, GER
Sodium dodecyl sulfate (SDS)	AppliChem, USA
Sodium hydroxide	Carl Roth, GER
Sulfosalicylic acid	Carl Roth, GER
Trichloroacetic acid	Merck, GER
Trifluoroacetic acid	Sigma, GER
Tris(hydroxymethyl)-aminomethan	AppliChem, USA
Triton X-100	Carl Roth, GER
Trypanblue	Promega, USA
Trypsin inhibitor, soybean	AppliChem, USA

Tween® 20	AppliChem, USA
Vasoactive intestinal peptide	Sigma, USA

3.2 Cells and cell culture

HEK293 cells (Human embryonic kidney cells, clone 293, adenovirus type 5 transformed)	DSMZ, GER
Dulbecco´s Modified Eagle´s Medium	Sigma, USA
G418	Calbiochem, USA
Fetal calf serum (FCS)	Biochrom, GER
L-Glutamine 200 mM	Sigma, USA
Penicillin / Streptomycin 100 IU/100 µg / ml	Biochrom, GER
Trypsin / EDTA 10x (0.5 % / 0.2 % w / v)	Biochrom, GER

3.3 Technical equipment and software

3.3.1 Technical equipment

Acrylamide gel casting stand	Mini-Protean® 3, BioRad, USA
Acrylamide gel electrophoresis cell	Mini-Protean® 3, BioRad, USA
Cell counter	Casy, Schäfe System, GER
Centrifuges	Mikro 20 Hettich, GER
	Z 233 MK Hermle, GER
Clean bench	Kojair®, Heraeus, GER
End-over-end shaker	Multishaker Rotator, RS-24, Biosan, GER
Films	X-OMAT films, Kodak, GER
Freezers	Liebherr comfort, Liebherr, GER
Fridge	Bosch, GER
Incubator with shaker (bacteria)	Innova 4230, New Brunswick Scientific, USA
Incubator for cell culture	Incusafe, Sanyo, GER

Gel dryer	Modell 583, BioRad, USA
Homogenisor	POTTER S, B. Braun Biotech, USA
Magnetic stirrer	RCT basic, IKA®,-Werke, GER
Microscopes	Laser Scanning Microscope-Zeiss 510 META; Carl Zeiss, GER
	Axiovert 40C, Zeiss, GER
PCR machine	Peltier Thermal Cycler PTC-200; MJ Research, USA
pH meter	TOLEDO MP220 Mettler, CH
Photometer	Gene Quant II, Pharmacia Biotech, GER
Pipettes	Eppendorf, GER
Power supply	Power Pac 3000, BioRad, USA
Rollator	RM5 superior, Marienfeld GmbH, GER
Scales	L2200P, Sartorius, GER
	Typ 1712, Sartorius, GER
Sequencer ABI 373 A	Perkin Elmer, USA
Shaker	Duomax 1030, Heidolph Instruments, GER
Sonicator	Sonopuls UW 2040, Bandelin
	Electronics, USA
Thermomixer	Thermomixer compact, Eppendorf, GER
Vortexer	VF2, Jahnke & Kunkel, GER
Water bath	WB 12, medingen, Preiss-Daimler, GER
Western blot cell	Mini Trans-Blot cell, BioRad, USA

3.3.2 Software

Clone Manager 3.0	Sci-Software
CorelDRAW 7.0	Corel Corporation
Corel Photo-Paint 7.0	Corel Corporation
DNAstar, SeqMan Pro	DNASTAR, Inc.
Excel 2000	Microsoft
Photoshop 7.1	Adobe
Power Point 2000	Microsoft
Prism GraphPad (version 3.0)	GraphPad Software Inc.
Word 2003	Microsoft
Zeiss LSM Image Browser 4.0	Car Zeiss Jena GmbH

3.4 Isolation and purification systems, kits

BigDye Terminator v1.1 Cycle Sequencing kit	Applied Biosystems, USA
ImProm-II™ Reverse Transcription system	Promega, USA
Invisorb® Spin Cell Mini kit	Invitek, GER
Invisorb® Spin Plasmid Mini kit	Invitek, GER
LigaFast™ Rapid DNA Ligation system	Promega, USA
Qproteome Cell Compartment kit	Qiagen, GER
QuikChange® Site-Directed Mutagenesis kit	Stratagene, USA
RNeasy® Plus Mini kit	Qiagen, GER
Ubiquitinated Protein Enrichment kit	Calbiochem, GER

3.5 Molecular standards

1kb DNA ladder	Invitrogen, USA
1kb DNA ladder	Fermentas, CAN
PeqGold protein marker IV, prestained	Peqlab, GER
Precision plus protein standards, dual color	BioRad, USA
Protein ladder, 10 - 200 kDa	Fermentas, GER

3.6 Radioactive labeled substances

[^{3}H]8-arginine vasopressin	Perkin Elmer (Boston, USA)
[^{3}H]cAMP	Perkin Elmer (Boston, USA)
[α-^{32}P]ATP	Perkin Elmer (Boston, USA)
EasyTag™ EXPRESS^{35}S Protein Labeling Mix	Perkin Elmer (Boston, USA)

3.7 Antibodies

3.7.1 First antibodies:

19S regulator, ATPase subunit Rpt1, rabbit polyclonal antibody (pAb)	Biomol, USA
19S regulator, non-ATPase subunit Rpn10, mouse monoclonal antibody (mAb)	Biomol, USA
20S proteasome α / β subunits, rabbit pAb	Biomol, USA
Calnexin, rabbit pAb	Stressgen, CAN
Derlin-1, rabbit pAb	Sigma, USA
ERGIC-53 (G1 / 93), mouse mAb	Axxora USA
ERGIC-53 / p58, rabbit pAb	Sigma, USA
ANTI-FLAG M2 Affinity Gel, mAb covalently attached to agarose	Sigma, USA
ANTI-FLAG polyclonal rabbit IgG	Sigma, USA
Anti-GAPDH, rabbit pAb	Cell Signaling Technology, USA
Anti-GM130, mouse mAb	BD Biosciences, GER
Anti-HA, mouse mAb, clone HA-7	Sigma, USA
Anti-HA agarose conjugate, clone HA-7	Sigma, USA
Hrd1 (C-term.), rabbit pAb	Abgent, USA
Multi-ubiquitin, mouse mAb	Stressgen, CAN
VCP, rabbit pAb	Cell Signaling Technology, USA
Anti-p44 / 42 MAP kinase, rabbit pAb	Cell Signaling Technology, USA
Anti-phospho- p44 / 42 MAP kinase, rabbit pAb	Cell Signaling Technology, USA

3.7.2 Secondary antibodies:

Alexa Flour 488 goat-anti-rabbit IgG	Invitrogen, USA
Cy-3 conjugated goat-anti-mouse IgG	Dianova, USA
Cy-3 conjugated goat-anti-rabbit IgG	Dianova, USA
Peroxidase-conjugated donkey anti-sheep IgG	Dianova, USA
Peroxidase-conjugated donkey anti-mouse IgG	Dianova, USA
Peroxidase-conjugated donkey anti-rabbit IgG	Dianova, USA

3.8 Enzymes

Endoglycosidase H (Endo H)	New England BioLabs Inc., UK
Peptide endoglycosidase F (PNGase F)	New England BioLabs Inc., UK
PfuTurbo® DNA polymerase	Stratagene, USA
Restriction enzymes, several	New England BioLabs Inc., UK

3.9 Vectors

pcDNA3.1	Invitrogen, USA
PEGFP N.1	Invitrogen, USA

3.10 Oligonucleotides (5’ to 3’)

3.10.1 Sequencing primers for the V2R

A3	GGG ACC CGC TGC TAG CC
B3	GGC TAG CAG CGG GTC CC
A4	CCG TGA AGT ATC TGC AG
B4	CTG CAG ATA CTT CAC GG
A5	CTG GGC CTG CTT TGC GG
B5	CCG CAA AGC AGG CCC AG
A6	GTG GTC GTC TAT GTG CTG
B6	GCA CAT AGA CGA CCA C

3.10.2 Sequencing primers for the pcDNA3.1 vector

T7 fw	TAA TAC GAC TCA CTA TAG GG
BGH bw	TAG AAG GCA CAG TCG AGG

3.10.3 Insertion primer for BamHI restriction site and N-terminal FLAG-tag in the V2R

V2R-flag fw	CGG GAT CCC GGC CAC CAT GGA CTA TAA GGA CGA TGA CGA TAA GAT GCT CAT GGC GTC CAC CAC TTC CGC

3.10.4 Insertion primer for BamHI restriction site and N-terminal HA-tag in the V2R

HA.V2R fw	CGG GAT CCC GGC CAC CAT GTA CCC ATA CGA TGT TCC AGA TTA CGC TAT GCT CAT GGC GTC CAC CAC TTC CGC

3.10.5 Mutagenesis primers for FLAG-tagged receptor mutants

L62P fw	GTG CTG GCG GCC CCA GCT CGG CGG
L62P bw	CCG CCG AGC TGG GGC CGC CAG CAC
K100R fw	GCC CCA GCT GGC CTG GAG GGC CAC CGA CCG CTT CCG
K100R bw	CGG AAG CGG TCG GTG GCC CTC CAG GCC AGC TGG GGC
K116R fw	CCC TGT GTC GGG CCG TGA GGT ATC TGC AGA TGG TGG G
K116R bw	CCC ACC ATC TGC AGA TAC CTC ACG GCC CGA CAC AGG G
S167L fw	GCT AGT GGC TTG GGC CTT CTT GCT CCT TCT CAG CC
S167L bw	GGC TGA GAA GGA GCA AGA AGG CCC AAG CCA CTA GC
G201D fw	GCG GAG CCC TGG GAC CGT CGC ACC
G201D bw	GGT GCG ACG GTC CCA GGG CTC CGC
V226E fw	GCC GCC TGC CAG GAG CTC ATC TTC C
V226E bw	GGA AGA TGA GCT CCT GGC AGG CGG C
K268R fw	GCA GCT GTG GCC CGG ACT GTG AGG ATG ACG C
K268R bw	GCG TCA TCC TCA CAG TCC GGG CCA CAG CTG C
K367R fw	GCC AGC TCC TCC CTG GCC CGG GAC ACT TCA TCG
K367R bw	CGA TGA AGT GTC CCG GGC CAG GGA GGA GCT GGC

3.11 Bacteria

3.11.1 Strains

Escherichia coli (E. coli) DH5-α genotype F-φ80lacZ ΔM15 Δ(lacZYA-argF) U169 endA1 recA1 hsdR17 (rk-,mk+) supE44 thi-1 gyrA96 relA1 phoA	Life Technologies, USA

3.11.2 Media and reagents

Luria-Bertani Medium (LB medium) (Tryptone 1 %, yeast extract 0.5 %, NaCl 1 % in dH_2O, pH 7.5)	Life Technologies, USA
Agar	Life Technologies, USA
Ampicillin sodium salt	Sigma, USA
Kanamycin	Sigma, USA
Tryptone	AppliChem, USA
Yeast extract	GIBCO, USA

3.11.3 Selection

Liquid culture

LB medium supplemented with ampicillin (100 µg / ml) or kanamycin (30 µg / ml).

Agar plates

LB medium supplemented with 1.5 % agar and ampicillin (100 µg / ml) or kanamycin (30 µg / ml).

4 Methods

All buffers were prepared with deionized water (dH_2O) and stored at 4 °C.

4.1 General methods of Molecular Biology

Standard DNA preparations and cloning techniques were carried out according to the handbook "Molecular Cloning" of Sambrook and Russel (Cold Spring Harbor Laboratory Press, 2001).

4.1.1 Preparation of competent cells

Reagents and solutions:

SOB medium	Tryptone	20 g
	yeast extract	5 g
	NaCl 5 M	2 ml
	KCl 2 M	1.25 ml
	dH_2O	990 ml
	autoclaved 20 min, 121 °C	
added before use (sterile filtered 0.2 µm):	$MgCl_2$ 2 M	10 ml
TB buffer (sterile filtered 0.2 µm):	PIPES	10 mM
	$CaCl_2$ x 2 H_2O	15 mM
	KCl	250 mM
	$MnCl_2$ x 4 H_2O	55 mM
	dH_2O	990 ml pH 6.75

The *E. coli* DH5-α genotype was selected for the production of competent cells to achieve high cloning efficiency. The method leads to holes in the bacterial membranes, which enables the competent cells to take up exogenous plasmid DNA.

Day 1

DH5-α bacteria were cultured on an agar plate without antibiotics in an incubator at 37 °C overnight.

Day 2

One single colony from the agar plate was picked and inoculated in 5 ml LB medium; the liquid culture was shaken in an incubator at 37 °C overnight (preparatory culture).

Day 3

20 µl of preparatory culture was used to inoculate 400 ml SOB medium at noon; the liquid culture was grown by shaking at 18 °C for 48 h.

Day 5

The liquid culture was controlled by measurement of the optical density (OD) in a photometer. The reference was SOB medium without bacteria. DH5-α bacteria were grown until the culture reached an OD of 0.4 – 0.6, which indicates the log-phase of exponential growth. They were harvested by centrifugation at 2,500 x *g* for 15 min at 4 °C. The pellet was resuspended in 140 ml ice-cold TB buffer and incubated for 10 min on ice. Cells were pelleted by a second centrifugation step (same conditions), the supernatant was removed and they were taken up in 34.6 ml TB buffer supplemented with 7 % DMSO. Cells were incubated for 30 min on ice and aliquots of 200 µl competent cells / microtube were directly frozen into a N_2 bath. Competent cells were stored at -80 °C.

4.1.2 Transformation of competent cells

Reagents and solutions:

$MgCl_2$ / $MgSO_4$ 2 M	$MgCl_2$	20.33 g
	$MgSO_4$	24.65 g in 100 ml dH_2O
Glucose solution 1 M	glucose	18 g in 100 ml dH_2O
SOC medium	tryptone	10 g
	yeast extract	2.5 g
	NaCl	300 mg
	KCl	250 mg
	dH_2O	485 ml pH 7.0
	autoclaved 15 min, 121 °C	
added (sterile filtered 0.2 µm):	$MgCl_2$ / $MgSO_4$ 2 M	5 ml
	glucose 1 M	10 ml

The purpose of this technique is to use bacteria to produce large quantities of plasmid DNA. Plasmid DNA carries a gene that encodes a resistance marker for an antibiotic. Only bacteria that have taken up the plasmids can survive under the selective pressure of antibiotics.

Competent cells (stored at -80 °C) were thawed on ice and aliquots of 50 µl competent cells were pipetted in pre-cooled microcentrifuge tubes. 50 nM plasmid DNA / sample was added and mixed carefully with the competent cells. After an incubation period of 30 min on ice, the transformation samples were heated for 45 s to 42 °C and placed for 2 min on ice for the DNA uptake. 400 µl SOC medium (without antibiotics, preheated to 42 °C) was added and the bacteria suspensions were incubated in an Eppendorf thermomixer at 37 °C for 30 min at 300 rpm. For selection 50 – 450 µl of bacteria culture were plated on an agar plate containing the appropriate antibiotic (ampicillin (100 µg / ml) or kanamycin (30 µg / ml)). The plasmid containing bacteria were grown in an incubator at 37 °C overnight.

4.1.3 Plasmid replication and isolation

Plasmid replication

One single colony of bacteria selected on a agar plate was used to inoculate 3 ml LB medium supplemented with antibiotics (depending on the plasmid, (ampicillin (100 µg / ml) or kanamycin (30 µg / ml)) and the liquid culture was shaken at 220 rpm in an incubator (37 °C) for 16 h. 2 ml suspension were transferred into a 2 ml microcentrifuge tube and bacteria were pelleted by centrifugation (13,000 x *g*, 1 min, RT), the supernatants were discarded. The pellets were stored at -20 °C or directly used for plasmid isolation.

Plasmid isolation

Transformed and amplified DNA has to be extracted and separated from bacterial components. The Spin Cell Mini kit from Invisorb provides filters and buffers to achieve purified plasmid DNA.

Bacteria pellets were resuspended in 200 µl resuspension solution by pipetting up and down. For plasmid isolation, 200 µl of alkaline lysis solution were added and the samples were mixed by carefully shaking the tubes 5 times. After a 4 min incubation, 200 µl neutralizing buffer was pipetted into the mixtures and the tubes were inverted 5 times. For separation of plasmids and bacterial debris, samples were centrifuged for 5 min at 16,000 x *g* and the supernatants were transferred to Spin filters placed in 2 ml microfuge tubes. After addition of 200 µl binding solution and inverting tubes one time, samples were centrifuged for 1 min at 6,000 x *g* and the plasmid DNA was isolated by absorbing on the Spin filter. The filtrates were discarded and the DNA was cleaned by addition of 750 µl wash buffer PL and subsequent centrifugation for 1 min at 6,000 x *g*. After removing of the filtrates, Spin Filters were centrifuged for 3 min at full speed to remove residual ethanol and were placed in new 1.5 ml microcentrifuge tubes. Addition of 70 µl dH_2O and an incubation for 1 min detached purified DNA from the filters and a final centrifugation step at 6,000 x *g* for 1 min eluted the plasmid DNA.

4.1.4 Plasmid construction of pFLAG.V2R and pHA.V2R with PCR

The FLAG-tag and the HA-tag are polypeptide protein tags that enable the purification and detection of recombinant fusion proteins. Plasmids pFLAG.V2R and pHA.V2R encoding the V2R with a FLAG-tag or a HA-tag fused to the N terminus, were constructed of a pcDNA3.1 vector containing the untagged V2R as template (Schülein, Liebenhoff et al. 1996; Oksche, Leder et al. 2002). The tags were fused to the V2R via PCR with BamHI / FLAG or a BamHI / HA insertion primers and the commercial available pcDNA3.1 / BGH reverse sequencing primer (sequences are shown in "Materials"). For the cloning reaction, the resulting PCR products and a pcDNA3.1 vector were digested with the restriction endonucleases BamHI and XbaI.

Reaction:

10x Pfu reaction buffer	5 µl
DNA template (50 ng)	1 µl
Primer FLAG.V2R fw 10 µM	0.6 µl
Primer BGH rev 5 µM	1.2 µl
dNTPs	1 µl
dH_2O	to 50 µl
Pfu-Turbo Polymerase	1 µl

Cycling parameters:

Cycles	**Temperature**	**Time**
1	95 °C	2 min
2-24	95 °C	30 s
	57 °C	1 min
	74 °C	1 min 20 s

4.1.5 Molecular cloning

4.1.5.1 Digestion with restriction enzymes BamHI and XbaI

Reaction mixture:

PCR product	30 µl	pcDNA3.1 vector	1 µg
NEB buffer 2	5 µl	NEB buffer 2	2 µl
NEB BSA 10 x buffer	5 µl	NEB BSA 10 x buffer	2 µl
BamHI	1.25 µl	BamHI	0.75 µl
XbaI	1.25 µl	XbaI	0.75 µl
dH_2O	ad 50 µl	dH_2O	ad 20 µl

PCR products (insert) and pcDNA3.1 vectors were digested at 37 °C for 1 h at 300 rpm in an Eppendorf thermomixer.

4.1.5.2 Agarose gel electrophoresis and gel extraction

Reagents and solutions:

TAE buffer	Tris	4.84 g
	acetic acid	1.197 g
	EDTA 0.5 M (pH 8)	2 ml
	dH_2O	ad 1 l
Electrophoresis buffer	ethidium bromide 1 %	5 µl
	TAE buffer	ad 100 ml

The size of the PCR products was determined by agarose gel electrophoresis on a 1 % agarose gel (in 100 ml TAE buffer supplemented with 5 µl ethidium bromide solution 1 %) at 80 mV. This method also separated the DNA fragments from the PCR mixture and the restriction enzymes.

FLAG.V2R and HA.V2R inserts and vector fragments of the correct size were cut out of the agarose gel with a scalpel and were directly frozen in 0.5 ml microcentrifuge tubes at -80 °C. After 10 minutes a small hole was engraved into the bottom of the tubes with help of a 19-gauge needle. The 0.5 ml tubes were placed in 1.5 ml tubes and quickly centrifuged at 16,000 rpm for 2 min. This process separated gel pieces from eluted DNA. The DNA fragments were directly used for ligation.

4.1.5.3 Ligation

The LigaFast™ Rapid DNA Ligation system contains a T4 DNA ligase that ligates vector and insert within an incubation time of 5 min at RT.

Reaction:

FLAG.V2R fragment (insert)	3.3 µl
vector fragment	0.7 µl
2 x Rapid ligation buffer	5 µl
T4 ligase	1 µl

4.1.5.4 Transformation and selection

The complete reaction mixtures of the ligation were transformed into competent DH5-α bacteria as described in 4.1.2 and plated out on agar plates supplemented with ampicillin (100 µg / ml). The next day, 20 colonies of bacteria were picked and 3 ml liquid LB cultures were inoculated for the following plasmid isolation (see 4.1.3). Plasmids were digested with the restriction endonucleases BamHI and XbaI as described above to control the insertion of the FLAG.V2R or the HA.V2R fragments. Insert-positive clones were verified by sequencing (see 4.1.8).

4.1.6 Site-directed mutagenesis of pFLAG.V2R and pHA.V2R

For the construction of plasmids pL62P, pS167L, pG201D and pV226E the QuikChange Site-Directed Mutagenesis kit of Stratagene was used according to the manufacturer's instructions with pFLAG.V2R as a template and the corresponding primers (see 3.10.5). pTet K (K100R, K116R, K268R, K367R), a lysine-free V2R mutant, was constructed analogical of pHA.V2R with four different PCRs (see primers in 3.10.5).

Reaction:

10x Pfu reaction buffer	5 µl
DNA template	30 ng
fw primer (10 µM)	125 ng
bw primer (10 µM)	125 ng
dNTPs	1 µl
dH_2O	ad 50 µl
Pfu-Polymerase	1 µl

Cycling parameters:

Cycles	**Temperature**	**Time**
1	95 °C	30 s
2-12	95 °C	30 s
	55 °C	1 min
	68 °C	6 min

4.1.7 GFP plasmids

Plasmid pEU367.GFP, encoding a C-terminally GFP-tagged V2R lacking the last three amino acids and plasmids containing the mutant receptors pL62P.GFP and pV226E.GFP have been described and characterized before.

For the construction of the plasmid pG201D.GFP the QuikChange Site-Directed Mutagenesis kit of Stratagene was used according to the manufacturer's protocol with pEU367.GFP as template and the corresponding primers (see 3.10.5).

4.1.8 Sequence analysis

All DNA plasmids were verified by sequencing. The Big Dye Terminator sequencing mix contains dideoxynucleotides using the Sanger chain termination method. After the PCR reaction, the sequencing products had to be purified from primers and sequencing mix by precipitation with ethanol.

Reaction:

DNA template (300 ng)	x µl
Big Dye Terminator	2.5 µl
Half Dye	5 µl
Primer (5 µM)	2 µl
dH_2O	ad 20 µl

Cycling parameters:

Cycles	Temperature	Time
1	94 °C	1 min
2-30	94 °C	16 s
	52 °C	16 s
	60 °C	2 min

Precipitation of sequencing samples

When the PCR was completed, the PCR mixture was first mixed with 2 µl 1.5 M sodium acetate / 250 mM EDTA buffer and then with 80 µl 95 % ethanol. After vortexing the mixture was incubated for 20 minutes on ice. For separation of precipitated DNA, the samples were centrifuged for 15 min at 13,700 x *g* at RT and supernatants were removed carefully. DNA pellets were covered with 400 µl 70 % ethanol, the samples were centrifuged again for 5 min (13,700 x *g*, RT) and supernatants were removed. The DNA pellets were air-dried at RT.

For capillary electrophoresis in the ABI Prism 310 Genetic Analyzer, DNA pellets were dissolved in 20 µl formaldehyde loading dye, incubated for 15 min and mixed by vortexing. Sequencing data analysis was done with the DNAstar SeqMan Pro software.

4.2 Cell culture and establishing of stably expressing HEK293 cell clones

4.2.1 Cell culture

HEK293 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % (v / v) heat-inactivated FCS, 20 mM glutamine and 100 IU of penicillin and 100 µg / ml streptomycin (complete medium) in a 5 % CO_2 atmosphere at 37 °C. The stably expressing HEK293 cell clones were additionally maintained with G418 (80 mg / ml). HEK293 cells were grown adherent and confluent cell layers were split after 3 - 4 days after seeding. For this, cells were incubated in 1 ml trypsin / EDTA solution (0.05 % / 0.02 %) for 4 min at 37 °C to detach them, followed by an addition of 5 ml complete medium to inactivate trypsin. Cells were counted in a semi-automatic cell counter; the seeding densities were 1 x 10^6 cells / 25 cm^2 flask or 60 mm dish, 2.5 x 10^6 cells / 100 mm dish and 2 x 10^5 cells per well of a 24-well plate. Cells were tested for mycoplasma every four weeks with the MycoAlert® Mycoplasma detection assay (Lonza, Switzerland).

4.2.2 Transient transfection with Lipofectamine™ 2000 transfection reagent

For the expression of recombinant proteins it is necessary that cells take up exogenous plasmid DNA. Cationic lipids and negatively charged DNA form a liposomal complex that can fuse with plasma and nuclear membranes. The plasmid DNA is then replicated together with the DNA of the host cell.

Cells were grown in a 35 mm culture dish until 90 % of confluence. The growth medium was replaced by 2 ml complete medium without antibiotics. 4 µg of the DNA were diluted in 250 µl of serum-free medium, 10 µl of Lipofectamine™ 2000 were mixed with additional 250 µl of serum-free medium and both incubated for 5 min. Both preparations were combined, mixed gently and incubated for further 20 min. The mixture was added in drops to the culture dish containing cells and medium. Cells were incubated for 24 - 48 h in a CO_2 incubator at 37 °C. The day after transfection, the medium was replaced by complete medium. Transfection in other culture vessels was carried out with amounts and volumes according to the following schedule:

Table 1: Transient transfection:

Culture vessels	Vol. of plating medium (µl)	DNA (µg) / media vol. (µl)	Lipofectamine™ 2000 (µl) in media vol. (µl)
24-well	500	0.8 / 50	2.0 / 50
35 mm	2000	4.0 / 250	10.0 / 250
60 mm	5000	8.0 / 500	20.0 / 500
100 mm	15000	24.0 / 1500	60.0 / 1500

4.2.3 Generating stably expressing cell clones

For stable transfection of cells the plasmid DNA has to be incorporated into the nuclear genome. This takes place in only few cases after transient transfection; the stable transfectants have to be selected by a resistance marker located on the incorporated plasmid. For selection G418 is commonly used, an aminoglycoside antibiotic that is toxic for cells without resistance. On the pcDNA 3.1 vector a *neo* gene encodes an aminoglycoside 3'-phosphotransferase, which inactivates the toxic antibiotic. Only cells that stably incorporate and express the resistance gene can survive in the presence of G418.

Cells were grown in 35 mm culture dishes until 90 % confluency. The growth medium was replaced by 2 ml complete medium without antibiotics. Transfection with Lipofectamine™ 2000 was carried out according to the manufacturer's instructions. After 48 h, the cells were treated with 500 µl trypsin / EDTA solution (0.05 % / 0.02 %) and incubated for 3 min at 37 °C. The trypsin / EDTA solution was inactivated by addition of 2 ml complete medium supplemented with G418 (80 mg / ml). Cells were transferred into a 60 mm culture dish containing 4 ml of complete medium supplemented with G418. After 24 h the cells that did not incorporated the plasmid in their genomic DNA started to die. G418 supplemented medium was changed every day. After one week most of the cells died and the remaining cells were detached and diluted with culture medium containing G418 to a volume of 8 ml (suspension A). In a second step, 1 ml of this suspension was diluted to a volume of 8 ml with complete medium (suspension B). The dilution series was performed in an analogous manner until suspension H. Cells were grown in two 24-well plates, in the first row 1 ml suspension A was transferred into each of the six wells and the rest of the suspension was discarded. In the second row 1 ml suspension B into each of the six wells was pipetted and so on. The complete medium supplemented with G418 was exchanged every second day. After two weeks single colonies of cell clones were visible in some wells. The single colonies were isolated and cultivated in new single wells until confluence. Then they were transferred to a culture vessel with larger cell growth area. Once the cells filled one 25 cm^2 flask, they were partially frozen and partially grown on glass cover slips to carry out an immunofluorescence analysis to

screen for different levels of protein expression. Only cell clones with homogenous protein expression were chosen.

4.2.4 RNA isolation and reverse transcription (RT)

Total genomic RNA was isolated from stably expressing cell clones to verify all V2R constructs. Total RNA was isolated from confluent 35 mm culture dishes with the RNeasy Plus Mini kit from Qiagen. RNA was reverse transcribed to cDNA by RT-PCR with the ImProm-II™ Reverse Transcription System of Promega. Subsequently, a PCR with specific V2R primers was carried out and the resulting DNA oligomer was the template for the sequencing reaction. The sequence of the V2R wild-type and all introduced mutations were confirmed in stably expressing cell clones by this method.

RT-PCR

For denaturation and hybridization of RNA and primers the reaction mixture was preheated to 70 °C for 5 min and immediately placed in ice-water bath for 10 min.

Hybridization reaction mix:

total RNA 1 µg / µl	1 µl
Oligo(dT)$_{15}$ primer 0.5 µg / µl	1 µl
nuclease-free H_2O	3 µl

For each sample 15 µl reverse transcription reaction mix was prepared, combined with 5 µl hybridization reaction mixtures and subsequently amplified by PCR.

Reverse transcription reaction mix:

ImProm-II™ 5x reaction buffer	4 µl
$MgCl_2$, 25 mM	2 µl
dNTP Mix, 10 mM	1 µl
Recombinant RNasin® Ribunuclease Inhibitor	0.5 µl
ImProm-II™ Reverse Transcriptase	1 µl
nuclease-free H_2O	6.5 µl

Cycling parameters:

Cycles	Temperature	Time
1	94 °C	2 min
2-25	94 °C	1 min
	60 °C	1 min
	72 °C	2 min
final extension	72 °C	5 min
hold	4 °C	

In addition, the cDNA of the V2Rs obtained by the RT-PCR was amplified by PCR using specific V2R primers. The products were controlled by agarose gel electrophoresis (see 4.1.5.2) and the mutations verified by sequencing analysis.

Reaction:

cDNA of the RT-PCR	1 µl
PCR 10x buffer	5 µl
A3 Primer (5 µM)	2 µl
B6 Primer (5 µM)	2 µl
dNTPs	1 µl
Taq-Polymerase (5 units / µl)	0.125 µl
dH_2O	ad 50 µl

Cycling parameters:

Cycles	Temperature	Time
1	95 °C	5 min
2-30	95 °C	0.5 min
	55 °C	0.5 min
	72 °C	1 min
final extension	72 °C	10 min
hold	4 °C	

4.3 Biochemical methods

4.3.1 Whole cell lysates

Reagents and solutions:

Phosphate buffered saline (PBS)	NaCl	137 mM
	KCl	2.7 mM
	Na_2HPO_4 x $2H_20$	8.1 mM
	KH_2PO_4	1.5 mM pH 7.4
RIPA stock solution	Tris-HCL pH 7.5	10 mM
	NaCl	100 mM
	Triton X-100	1 %
	sodium deoxycholate	1 g
	SDS	0.1 %
	dH_2O	ad 100 pH 7.5
Protease inhibitors mix	trypsin inhibitor	2.0 µg / ml
	aprotinin	1.0 µg / ml
	benzamidine	100 mM
PMSF stock solution	PMSF	40 mM in ethanol
RIPA buffer	RIPA stock solution	10.0 ml
	protease inhibitors mix	86 µl
	PMSF stock solution	125 µl

Cells were grown in 60 mm culture dishes until confluence. After aspiration of the culture medium and two washing steps with phosphate buffered saline (PBS), cells were covered with 500 ml of RIPA buffer, scraped and collected in a 1.5 ml micro centrifuge tube (pre-cooled at 4 °C). Cell membranes were disrupted by treatment with the sonicater (3 times for 1 s). After centrifugation at 18,600 x *g* for 20 min (4 °C), 450 µl of supernatant was collected in a new microcentrifuge tube and used for protein quantification or stored at -20 °C.

4.3.2 Determination of protein concentration

Reagents and solutions:

Coomassie reagent (Bradford reagent)	Coomassie brilliant blue G250	100 mg
	Ethanol 96 %	50 ml
	Phosphoric acid 85 %	100 ml
	dH_2O	ad 1 l
Bovine serum albumin (BSA) 1.0 mg / ml	albumin standard Pierce 2.0 mg / ml	500 µl
	dH_2O	500 µl
2 N NaOH	NaOH	80 g
	dH_2O	ad 1 l
RIPA buffer	see 4.3.1	

The quantification of the total amount of proteins was assessed by the colorimetric method of Bradford. The dye Coomassie Brilliant blue G250 binds proteins in an acidic solution; this leads to a shift of the maximum of absorbance from 465 nm to 595 nm causing a visible color change that can be measured with a photometer.

The samples were prepared in duplicates and compared with a BSA standard curve. 5 µl whole cell lysates of unknown protein concentration were diluted with 45 µl dest. H_2O and 50 µl 2 M NaOH. The BSA standard curve was set up by mixing different amounts of a BSA stock solution (1 mg / ml) with dest. H_2O to 45 µl, 5 µl of the RIPA buffer and 50µl of 2 M NaOH. The unknown protein concentration has to be within the range of the BSA standard curve; a series of 0, 5, 10, 20, 30 and 40 µg BSA was considered valid. Both samples and standard mixtures were heated to 60 °C for 10 min, after 2 min at RT they were mixed with 1.0 ml of the Bradford reagent and the absorption was measured in a 96-well plate at 620 nm. The protein concentration was interpolated using 3-parameter polynomial equation of the standard curve.

4.3.3 Subcellular fractionation

Proteins of different cellular compartments can be obtained with specific extraction buffers and centrifugation steps; this leads to separation of cytosolic, membrane and nuclear fractions.

Different subcellular protein fractions were isolated with the Qproteome Cell Compartment kit (Qiagen, Germany).

Reagents and solutions of the Qproteome cell compartment kit:

	µl / 4.5 x 10^6 HEK293 cells	protein fraction
Extraction buffer CE1	1000	cytosol
Extraction buffer CE2	1000	membranes
Extraction buffer CE3	500	nucleus
Benzonase® nuclease	7	
Protease inhibitor solution 100x	10 µl / 1000 µl extraction buffer CE1-3	
PBS	see 4.3.1	

HEK293 cells stably expressing V2Rs were incubated with 20 µM MG132 for 16 h or left untreated. 4.5 x 10^6 cells were transferred into a 15 ml Falcon® tube, centrifuged at 500 x *g* for 10 min at 4 °C and the supernatants were discarded. The cell pellets were resuspended in 2 ml ice-cold PBS, transferred into 2 ml microcentrifuge tubes, collected by centrifugation at 500 x *g* for 10 min at 4 °C and the supernatants were removed. The washing step was repeated and the cell pellets were resuspended in 1 ml ice-cold extraction buffer CE1 and incubated for 10 min at 4 °C. After centrifugation (1,000 x *g*, 10 min, 4 °C) the supernatants of fraction CE1 contained the cytosolic proteins. The pellets were resuspended in 1 ml ice-cold extraction buffer CE2 and incubated for 30 min (4 °C). The samples were centrifuged (6,000 x *g*, 10 min, 4 °C) again and the supernatants CE2 primarily contained membrane proteins. To isolate nuclear proteins, the pellet was spiked with 13 µl dH_2O and 7µl Benzonase® nuclease for 15 min at RT. Nuclear proteins were extracted by addition of 500 µl extraction buffer CE3 for 10 min (4 °C) and the insoluble material was pelleted by centrifugation (6,800 x *g*, 10 min, 4 °C). Supernatants CE3 contained the nuclear proteins.

Equal amounts of proteins were used for SDS-PAGE. The purity of the fractions was controlled by detection of calnexin and GAPDH that are exclusively expressed in membrane and cytosolic compartments, respectively. Concurrently these proteins served as a loading control.

4.3.4 Immunoprecipitation

Reagents and solutions:

Buffer A	Tris-HCl pH 8.0	50 mM
	NaCl	150 mM
	EDTA pH 8.0	1 mM
	Triton X-100	1 % (w / v)
	SDS	0.1 % (w / v)

Lysis buffer A	Buffer A	10.0 ml
	protease inhibitors mix (see 4.3.1)	86 µl
	PMSF stock solution (see 4.3.1)	125 µl
Washing buffer I	Tris-HCl pH 8.0	50 mM
	NaCl	500 mM
	EDTA pH 8.0	1 mM
	Triton X-100	0.5 % (v / v)
	SDS	0.1 % (w / v)
Washing buffer II	Tris-HCl pH 8.0	50 mM
	EDTA pH 8.0	1 mM
	Triton X-100	0.5 % (v / v)
	SDS	0.1 % (w / v) pH 7.4
PBS	(see 4.3.1)	

Confluent cells in 60 mm culture dishes were placed on ice and washed twice with 1 ml of PBS. They were covered with 1 ml of ice-cold lysis buffer A and shaken for 10 min on ice. Cell lysates were collected in microcentrifuge tubes, mixed by vortexing and centrifuged at 13,700 rpm for 20 min at 4 °C. Supernatants were transferred to protein A-Sepharose beads (5 mg / 60 mm dish of cells) previously loaded with 5 µg of an anti-FLAG antibody (Sigma). The incubation on an end-over-end shaker was carried out from 2 h to overnight. The immunoprecipitated proteins were washed three times with 1 ml washing buffer I and one time with 1 ml washing buffer II. They were taken up in 40 µl 2x Roti-load 1, carefully mixed by vortexing, denaturated for 5 minutes at 95 °C and separated by SDS-PAGE.

4.3.5 Gel electrophoresis and western blot analysis

Reagents and solutions:

Separation gel buffer	Tris-HCl pH 8.8	0.75 M
Stacking gel buffer	Tris-HCl pH 6.8	0.625 M
Running buffer	Tris-OH	0.025 M
	glycine	0.19 M
	SDS	0.1 %
Blotting buffer	Tris-OH	0.02 M
	glycine	0.15 M
	methanol	20 % (v / v)
	SDS	0.015 % (w / v)

PBS-T 0.1 %	Tween 20	0.1 %
	PBS (see 4.3.1)	ad 1.0 l
Blocking buffer	low fat milk powder	5 % (w / v)
	PBS-T 0.1 %	ad 100 ml
Ponceau S stock solution	Ponceau S	2 %
	sulfosalicylic acid	30 %
	trichloracetic acid	30 %
Ponceau S staining solution	Ponceau S stock solution	0.1 %
PBS-T 1 % (antibodies)	low fat milk powder	2 % (w / v)
	PBS-T 0.1 %	ad 100 ml

Reaction mixture for gels:

2 Gels:	Separation gel 10 %:	Separation gel 15 %:	Stacking gel:
dH_2O	1.75 ml		3.5 ml
Rotiphorese Gel (30 % AA / 0.8 % BisA)	3.75 ml	5.625 ml	835 µl
Separation gel buffer	5.625 ml	5.625 ml	
Stacking gel buffer			625 µl
20 % SDS	56.5 µl	56.5 µl	25 µl
TEMED	5.65 µl	5.65 µl	5 µl
ammonium persulfate 10 %	79 µl	79 µl	25 µl

The separation gel was mixed according to the schedule, covered with isopropanol and left to polymerize for 45 min. After removing of the isopropanol, the stacking gel was prepared and casted upon the separation gel. Samples were denatured by Roti-load 1 loading buffer for 5 min at 95 °C and were loaded on the polymerized gel (size 5.8 x 8.1 cm). SDS-PAGE was carried out at 20 mA / gel to separate denatured protein / SDS complexes with the Mini-PROTEAN® 3 system of BioRad; the time was dependent on the molecular mass of the protein of interest. Molecular weight markers of Invitrogen, BioRad or Peqlab were used, depending on the protein to be analyzed.

After separation, proteins were blotted (4 °C, 99 mA / gel, time was dependent on the molecular mass of the protein of interest) for immunodetection onto nitrocellulose membranes using the Mini Trans-Blot Electrophoretic Transfer Cell from BioRad. Transfer was controlled by staining the molecular weight marker; for this the nitrocellulose membrane was incubated in Ponceau S staining solution for 30 s and subsequently decolorized by washing three times for 5 min with dH_2O. Nitrocellulose membranes

were blocked (1 h, RT) with blocking buffer to avoid unspecific binding of antibodies. The first antibody was diluted in PBS-T (0.1 % Tween 20, 2 % low fat milk powder, antibody concentrations are listed in the following schedule) and incubated at least 2 h (RT) or overnight (4 °C). Membranes were washed with PBS-T 0.1 % (3 x 10 min) and treated with a secondary antibody coupled with horseradish peroxidase (POD) (diluted 1:10,000 in PBS-T; 0.1 % Tween 20, 2 % low fat milk powder, depends on the origin of the first antibody) for 45 min (RT). Blots were washed two times for 15 min and one time for 30 min with PBS-T 0.1 %. Finally, membranes were incubated for 3 min in ECL luminescence solution (Applichem) and developed using a Kodak X-Omat film.

List of concentrations and origin of the first antibodies:

name:	company:	dilution:	species:
Anti-FLAG Sigma M2	Sigma	1:1,500	mouse
Anti-FLAG rabbit	Sigma	1:1,500	rabbit
Anti-Derlin-1	Sigma	1:2,000	rabbit
Anti-VCP	Cell Signaling	1:1,000	rabbit
Anti-multi-ubiquitin	Stressgen	1:400	mouse
Anti-Hrd1	Abgent	1 :1,000	rabbit
Anti-GAPDH	Cell Signaling	1:1,000	rabbit
Anti-calnexin	Sressgen	1:8,000	rabbit
Anti-19S reg. sub. Rpt1	Biomol	1:1,000	rabbit
Anti-19S reg. sub. Rpn10	Biomol	1:1,000	mouse
Anti-20S α / β subunits	Biomol	1:2,000	rabbit
Anti-HA	Sigma	1:1,000	mouse
Anti-p42 / 44 MAP kinase	Cell Signaling	1:1,000	rabbit
Anti-phospho-p42 / 44 MAP kinase	Cell Signaling	1:1,000	rabbit

4.3.6 Saturation binding experiments with [^{3}H]AVP

Binding experiments were performed to characterize the pharmacological ligand binding capacity of the wild-type V2R and to determine the number of specific binding sites. To eliminate non-specific hormone binding, the experiments were performed in presence (unspecific binding) or absence (total binding) of an excess of unlabeled hormone. The part of [^{3}H]AVP that can be displaced from specific binding sites by unlabeled AVP was taken into account for data analysis. The difference between total ligand binding and unspecific binding is the saturation binding. Saturation means that this differ-

ence reaches a plateau with increasing amounts of [^{3}H]AVP. The unspecific binding increases with the ligand concentration and is not saturable.

Reagents and solutions:

DPBS	$CaCl_2$ x 2 H_2O	0.133 g
	$MgCl_2$ x 6 H_2O	0.1 g
	KCl	0.2 g
	KH_2PO_4	0.2 g
	NaCl	8.0 g
	NaH_2PO_4	1.15 g
	H_2O	ad 1 l
NaOH	0.1 M in H_2O	

2 x 10^5 HEK293 cells stably expressing FLAG.V2R fusion proteins were grown in a poly-L-lysine coated 24-well plate for 24 h. Cells were washed twice with 2 ml DPBS and were incubated in a final volume of 300 µl DPBS supplemented with increasing amounts of [^{3}H]AVP (0.195 - 100 nM) in the absence (total binding) or presence of 1 µM unlabeled AVP (unspecific binding) for 2 h to equilibrate the balance of ligand-receptor complexes / unspecific binding and unbound hormone. The experiments were done at 4 °C to avoid receptor internalization. In addition, two wells of cells were harvested for protein quantification and two for determination of cell count. Subsequently, the labeling solution was aspirated and the cells were quickly rinsed 3 times with 1 ml ice-cold DPBS and the washing solution was removed. Cell lysis was carried out by incubation in 500 µl preheated 0.1 M NaOH / well at 37 °C for 45 min. The supernatants were transferred to scintillation vials, mixed with 4 ml liquid scintillator (Aquasafe 300 plus, Zinsser, Germany) and the radioactivity was determined in a liquid scintillation counter. Data analysis was done with the Prism GraphPad software (version 3.0); dissociation constant (K_D) and maximal binding (B_{max}) were calculated from specific binding isotherms. Cell count and protein concentration were used to calculate the amount of specific hormone binding sites by non-linear regression analysis.

4.3.7 Adenylyl cyclase activity assay

The activation of V2Rs by their ligand AVP leads to a signaling cascade, which is transmitted by the stimulating G protein Gs via the transmembrane residing adenylyl cyclase (AC) to further intracellular downstream messengers.

The activity of the adenylyl cyclase was measured by its catalyzed enzymatic reaction, which is the generation of the second messenger cAMP out of ATP. In the assay, the

generated [^{32}P]cAMP was isolated based on the two-column method of Salomon et al. (1974).

Reagents and solutions:

Membrane preparation		
Homogenization buffer	sucrose	27 % (w / v)
	EDTA	1 mM
	HEPES	20 mM pH 7.8
Resuspension buffer (RP)	EDTA	1 mM
	HEPES	20 mM pH 7.8
PBS	see 4.3.1	
AC activity assay		
RP / 2 % BSA	BSA	2 %
	EDTA	1 mM
	HEPES	20 mM pH 7.8
REA mix	Tris-HCl 1 M	1500 µl
	$MgCl_2$ x 6 H_2O 200 mM	600 µl
	EDTA 100 mM	600 µl
	10 mM IBMX	3000 µl
	100 mM cAMP	300 µl
	10 mM ATP	300 µl
	10 mM GTP	30 µl
	creatine phosphokinase C-3755	39.6 mg
	phosphocreatine P-7936	153 mg
	BSA	180 mg
	H_2O	2.67 ml
Stopping solution	ATP	4 mM
	cAMP	1.4 mM
	SDS	2 %
	[^{3}H]cAMP	1.75 mM in dH_2O
Imidazol-HCl buffer	imidazol	6.8 g
	dH_2O	ad 1 l pH 7.4

Membrane preparation

Nuclei-free crude membrane fractions were prepared from HEK293 cells stably expressing FLAG-tagged V2Rs and mutant receptors. 3 x 10^6 cells were grown in 100 mm culture dishes until confluence. Cells were washed twice with 5 ml ice-cold PBS, scraped in 2 ml PBS and transferred to a 15 ml Falcon® tube. They were pelleted by centrifugation (400 x *g*, 5 min, 4 °C), the supernatants were discarded and the cells were resuspended in 2 ml homogenization buffer. Cells were disrupted with 10 strokes at 750 rpm in a Potter S homogenizor and the suspension was transferred to microcentrifuge tubes and centrifuged at 20,000 x *g* for 10 min (4 °C). The supernatant was removed and the pellet was taken up in 500 µl resuspension buffer. 15 µl of the membrane suspension were used for protein quantification and the isolated crude membrane fractions were stored at -80 °C or directly utilized for the AC activity assay.

AC activity assay

Many factors affect enzyme activity; therefore it is necessary to use a special buffer system (Rea mix) and control samples in the assay for optimal results. The Rea mix contains an ATP regenerating system of phosphocreatine and creatin kinase, because changes in the initial ATP concentration may influence AC activity. IBMX is an inhibitor of phosphodiesterases, which may be contaminations of the membrane preparation. Phosphodiesterases catalyze the turnover of cAMP. Additionally, unlabeled cAMP is added to the reaction mixture to protect the generated [^{32}P]cAMP. In the control samples, forskolin stimulates all cellular adenylyl cyclases and vasoactive intestinal peptide (VIP) activates the stimulating G protein G_s to ensure a working enzyme and G protein, respectively. This is important to control, because not all tested mutants are able to activate the AC.

Crude membrane fractions were diluted to a concentration of 1 g protein / ml in RP / 2 % BSA buffer. The reaction mix was prepared to a volume of 80 µl / column on ice; it contained 30 µl Rea mix, 20 µl dH2O, 20 µl [α-^{32}P]ATP 0.75 µCi and 10 µl additives (diluted in RP / 2 % BSA, AVP (0, 0.03, 0.3, 1, 3, 30, 100, 1000 nM for wild-type V2R, and 1000 nM for all mutant receptors), Forskolin (100 µM) or VIP (1 µM). The reaction was started with the addition of 20 µl membrane proteins, incubated at 32 °C in a water bath and terminated after 20 min. After the end of the incubation period, the reaction was stopped with 500 µl stopping solution.

The [^{32}P]cAMP formed through the enzymatic reaction of the AC was separated and purified by a two column method. 600 µl sample were loaded on the first column containing Dowex 50 – X8, a cationic exchange resin that is negatively charged and absorbs [^{32}P]cAMP in a non-specific interaction (separation from negatively charged ATP, ADP, AMP, phosphate). The column was rinsed with 1 ml and 1.8 ml dH_2O, respec-

tively and eluted with 5 ml dH_2O. The Dowex-eluate was purified by a column filled with neutral aluminium oxide (Alox). Aluminium oxide binds [^{32}P]cAMP and also impurities of ATP. It was washed with 2 ml imidazol-HCl buffer and the [^{32}P]cAMP that is bound to a less extent then ATP, was eluted with 3 ml imidazol-HCl buffer. The eluates of Alox were transferred to scintillation vials, mixed with 13 ml liquid scintillator (Aquasafe 300 plus, Zinsser, Germany) and the radioactivity of [^{32}P]cAMP and of the column loading control [^{3}H]cAMP were determined using a liquid scintillation counter. The activity was determined with the following formula:

$$[\text{cAMP}] = \frac{[^{32}\text{P}] \times T \times 10000}{[^{3}\text{H}] \times P \times \text{Prot} \times 20\ \text{min}}$$

[^{32}P] represents the measured activity of [^{32}P] in cpm and T is the amount of tritium which was subjected to the assay. [^{3}H] is the amount of eluted tritium / column in cpm, P represents the input of [^{32}P] / column in cpm and Prot means the quantity of total proteins inserted per column (mg). EC50 was calculated from the resulting curve by using the Prism GraphPad software and non-linear regression.

4.3.8 Ubiquitination assay with ubiquitinated protein enrichment beads

Substrate proteins are covalently modified at lysine or cysteine residues by the 8 kDa ubiquitin molecule or by multi-ubiquitin chains of 2 - 8 ubiquitin moieties attached by ubiquitin ligases. Because of the rapid degradation of polyubiquitinated substrates, detection of these substrates should be performed in presence of the proteasome inhibitor MG132, which blocks the proteolytic activities of the catalytic core of the 26S proteasome and accumulates polyubiquitinated proteins.

Reagents and solutions:

Buffer A	(see 4.3.4)	
Lysis buffer B	Buffer A	10.0 ml
	protease inhibitors mix (see 4.3.1)	86 µl
	PMSF stock solution (see 4.3.1)	125 µl
	N-ethylmaleinimide 1 M solution	100 µl
Washing buffer I	(see 4.3.4)	
Washing buffer II	(see 4.3.4)	
TBS	Tris-HCl	10 mM
	NaCl	150 mM

TBS-T	Tween 20	0.1 %
	TBS	ad 1.0 l
Blocking/antibody buffer	low fat milk powder	5 %
	TBS-T 0.1 %	ad 100 ml
MG132 solution	MG132	4 mM in DMSO

Stably expressing HEK293 cells were incubated in the presence of 20 µM MG132 in serum-free medium for 16 h. Total protein lysis, as described in 4.3.4 for immunoprecipitation, was carried out with 1 ml lysis buffer B and the insoluble fraction was removed by centrifugation (20 min, 4 °C, 12,000 g). The supernatant was mixed with 60 µl of polyubiquitin enrichment beads to isolate all polyubiquitinated proteins for 4 h at 4 °C in an end-over-end shaker. The immunoprecipitated proteins were washed three times with 1 ml washing buffer I and one time with 1 ml washing buffer II. They were taken up in 40 µl 2x Roti-load 1, denaturated for 5 min at 95 °C and separated by SDS-PAGE for 1 h 15 min at 20 mA / gel.

After blotting on a nitrocellulose membrane (3 h, 99 mA / gel), proteins were dyed with Ponceau S solution. The molecular weight marker proteins were plotted and the membrane was destained 3 x 10 min with TBS-T 0.1 % and blocked for 30 min in blocking buffer and washed 4 x 10 min in TBS-T 0.1 %. The membrane was incubated with an anti-FLAG M2 antibody (Sigma) at a dilution of 1:1500 in TBS-T 0.1 % supplemented with 5 % milk powder and washed 4 x 10 min in TBS-T 0.1 %. The secondary POD-coupled antibody was used at a 1:10,000 dilution in TBS-T 0.1 % (5 % milk powder). The blot was washed twice for 10 min and once for 30 min in TBS-T 0.1 % and developed with the ECL system.

4.3.9 Digestion with the glycosidases Endo H and PNGase F

The glycosidases endoglcosydase H (Endo H) and peptide-N-(N-acetyl-β-glucosaminyl) asparagine amidase F (PNGase F) are used to characterize the N-glycosylation state of proteins. Endo H and PNGase F cleave off N-linked sugar modifications of proteins. Endo H removes only high-mannose residues from glycoproteins indicating of localization in compartments before the cis-Golgi network where N-glycycans become resistant to the action of Endo H. PNGase F cleaves off all types of N-glycosylation.

Cell lysates (described in immunoprecipitation 4.3.4) of 60 mm culture dishes were immunoprecipitated with 40 µl anti-FLAG affinity gel (Sigma) / dish. Immunoprecipitated proteins were washed (see protocol in 4.3.4) and at the last washing step the beads were divided into 3 equal aliquots. One sample was incubated without enzymes as a control, the others with Endo H or PNGase F to monitor size-shift, respectively. The digestion reactions were prepared according to the protocol:

Reaction mixture:

Reaction	**Endo H**	**PNGase F**	**Control**
PBS to suspend the beads	20.25 µl	18 µl	26 µl
Denaturation buffer	2.25 µl	2 µl	
	10 min, 95 °C		
G5 buffer / G7 buffer	2.5 µl	2.5 µl	
NP-40		2.5 µl	
Endo H / PNGase F	1 µl	1 µl	
	1 h, 37 °C		
4 x Roti-load 1	9 µl	9 µl	9 µl
	5 min, 95 °C		

Proteins were subsequently separated by SDS-PAGE and blotted onto nitrocellulose membranes for immunodetection with a monoclonal mouse anti-FLAG antibody (1:1500) and a POD-coupled anti-mouse antibody (1:10,000). Blots were developed using the ECL system.

4.3.10 Detection of mitogen-activated protein kinases

Serum-starved HEK293 cells stably expressing the V2R were analyzed for the ability to activate the MAPK pathway with different ligand concentrations. Cells were incubated with different amounts of AVP (0, 10, 50, 100, 500, 100 nM) for 10 min and total cell lysis was performed. Equal amounts of proteins were separated by SDS-PAGE and subjected to immunoblotting. The unphosphorylated and the active, phosphorylated forms of ERK1/2 were detected with anti-p42 / 44 MAP kinase and anti-phospho-p42 / 44 MAP kinase antibodies, respectively.

4.3.11 Pulse-chase assay

Reagents and solutions:

Chase medium	methionine	2.3 mM
	cysteine	0.75 mM
	complete medium	4 ml
Stop medium	complete medium	10.0 ml
	protease inhibitors mix	86 µl
	PMSF stock solution	125 µl
Lysis buffer A	(see 4.3.4)	
MG132 solution	MG132	4 mM in DMSO
Chloroquine solution	chloroquine diphosphate salt	5 mM in dH2O

For the determination of the degradation kinetics, newly synthesized receptors were metabolically labeled with [^{35}S] methionine and [^{35}S] cysteine and analyzed during a time frame.

Confluent 100 mm culture dishes of HEK293 cells stably expressing the receptor constructs were starved in 5 ml medium lacking the amino acids methionine and cysteine overnight (16 h). They were washed twice with PBS (37 °C), separated by treatment with 1 ml trypsin / EDTA solution (0.05 % / 0.02 %) at 37 °C for 3 min and the cell suspension was transferred into a 15 ml Falcon® tube. Cells were washed with PBS (37 °C) two additional times and were collected by centrifugation (5 min, 1000 x g, RT). The cell pellet was resuspended in 500 µl medium lacking methionine and cysteine and incubated with 220 µCi of EasyTag™ EXPRESS^{35}S Protein Labeling Mix for 45 min at 37 °C in a CO_2 incubator. The incorporation of radiolabeled amino acids was stopped with 4 ml of chase medium containing an excess of unlabeled methionine and cysteine. At time points 0, 2.5, 5 and 10 h the cells were resuspended and 1 ml cell suspension was harvested by transferring in 750 µl ice-cold stop buffer, centrifuged (5 min, 200 x *g*, 4 °C) and the supernatant was discarded.

Cell pellets were dissolved in 500 µl lysis buffer A, an immunoprecipitation with FLAG M2 antibodies coupled to protein A-Sepharose beads followed, and a subsequent SDS-PAGE was performed as described in 4.3.5. After gel electrophoresis, gels were dried on a gel dryer (2 h, 80 °C) and developed by autoradiography through exposure to Kodak X-ray films. When indicated, the autoradiographic signals were increased by a BioMax TRANSCREEN LE intensifying screen (Kodak). In experiments where protein turnover was blocked, all buffers contained specific inhibitors of protein degradation, starting from the labeling step until the end of the chase. Proteasomal inhibition was carried out by treatment with 40 µM MG132 and blocking of lysosomal enzymes was achieved in presence of 100 µM chloroquine.

4.3.12 Protein identification by mass spectrometry (MS)

For the identification of new interaction partners of the ubiquitinated wild-type V2R, capillary liquid chromatography tandem mass spectrometry (LC-MS/MS) experiments were performed.

Reagents and solutions:

Coomassie G250 solution	Comassie Brilliant Blue G250	0.08 %
	ortho-phosphoric acid	1.6 % (v / v)
	ammonium sulfate	8 %
	methanol	20 %

Gel washing buffer	acetonitrile	50 % (v / v)
	ammonium bicarbonate 25 mM	ad 100 ml
Digestion buffer	ammonium bicarbonate	5 mM
Extraction buffer	trifluoroacetic acid	0.5 %
	acetonitrile	ad 100 ml

Sample preparation

Confluent 60 mm culture dishes of HEK293 cells stably expressing FLAG.V2R fusion proteins were treated with 20 µM MG132 in serum-free medium for 16 h, the receptors were immunoprecipitated with 40 µl covalent-coupled FLAG affinity gel / dish and subjected to SDS-PAGE as described in 4.3.4 and 4.3.5. After Coomassie staining, the protein bands were excised, washed with gel washing buffer, shrunk by dehydration in acetonitrile, and dried in a vacuum centrifuge. The dried gel pieces were incubated with 60 ng trypsin (sequencing grade; Promega, Madison, WI, USA) in 10 µL digestion buffer at 37 °C overnight. To extract the peptides, 10 µL 0.5 % (v / v) trifluoroacetic acid in acetonitrile was added, the separated supernatant was dried under vacuum, and was redissolved in 6 µl 0.1 % (v / v) TFA in (6:94, v / v) acetonitrile–water. LC-MS/MS analysis was performed on a Micromass CapLC liquid chromatography system and a quadrupole orthogonal acceleration time-of-flight mass spectrometer Q-TOF Ultima (Micromass, Manchester, UK) equipped with a Z-spray nanoelectrospray source. LC-separations were performed on a capillary column (PepMap C18, 3 µm, 100 Å, 150 mm x 75 µm i.d., Dionex, Idstein, Germany). Data was acquired in a data-dependent mode using one MS scan followed by MS/MS scans of the most abundant peak. The processed MS/MS spectra (MassLynx version 4.0 software) and the MASCOT server version 1.9 (Matrix Science Ltd., London, UK) were used to search against the Swiss-Prot database (210906, 234.112 sequences). The maximum of two missed cleavages was allowed and the mass tolerance of precursor and sequence ions was set to 0.1 and 0.2, respectively. A protein was accepted as identified if the total MASCOT score was greater than the significance threshold and at least 2 peptides appeared the first time in the report and were the first ranking peptides.

4.4 Microscopic Methods

Reagents and solutions:

Poly – L – lysine stock solution	poly – L – lysine 5 mg Sigma	dissolved in 50 ml water
Working solution	poly – L – lysine stock solution	10 ml
	dH_2O	30 ml
PBS	see 4.3.1	

Preparation for the confocal laser-scanning microscopy

The microscopic methods require HEK293 cells growing on poly-L-lysine-coated glass cover slips (Ø 25 mm or 12 mm). The glass cover slips were first sterilised (5 h, 180 °C) and then transferred to 35 mm culture dishes or to wells of 24-well plates, respectivly. The working solution of poly-L-lysine had to cover the complete glass plate over 30 minutes, afterwords the solution was aspired and the coated platelets were air dried at least for 1 h.

4.4.1 Live cell imaging

5 x 10^6 HEK293 cells stably expressing GFP-tagged wild-type and mutant V2Rs were grown for 24 - 48 h in a 35 mm culture dish containing a poly-L-lysin coated glass cover slip (Ø 25 mm). They were treated with 150 nM LysoTracker Red for 1 h to stain lysosomal vesicles. Protein degradation was inhibited by additional incubation with 100 µM chloroquine for 3 h, an inhibitor for lysosomal enzymes. For microscopic studies the cells were transferred into a heating chamber and covered with 1 ml PBS. The GFP and LysoTracker Red flourescence were visualized on a Zeiss 510 invert confocal laser-scanning microscope (optical section: < 0.9 µm; multitrack mode; GFP, λ_{exc}: 488 nm, BP filter: 494 – 548 nm; LysoTracker® Red, λ_{exc}: 543 nm, LP filter: 560 nm) and an overlay of both signals was computed.

4.4.2 Immunofluorescence

This method enables immunostaining of proteins in paraformaldehyde-fixed cells. Co-staining of special compartments leads to the ascertainment of the localization of the protein of interest.

For immunofluorescence studies 2 x 10^5 HEK293 cells stably expressing the FLAG-tagged V2R and mutant receptors were spread out on poly-L-lysine-coated glass cover slips (Ø 12 mm) in a 24-well plate. Cells were grown for 24 h, washed twice with PBS (37 °C) and fixed for 25 min at RT in fixing buffer. The fixed cells were rinsed three times with PBS for 2 min on ice. When required, cell membranes were permeabilized with PBS supplemented with 0.075 % Triton X-100 (3 min) and were washed three

times with PBS (4 °C). For the incubation with the first antibody, which recognizes the protein of interest or a marker protein of an intracellular compartment, the glass cover slips were transferred into a moist chamber (box with water-soaked filter paper, pre-heated to 37 °C). The antibodies were diluted in PBS to a final concentration from 1:50 to 1:1000 and each glass cover slip was covered with 40 µl antibody solution and incubated for 45 min at 37 °C in the moist chamber. Glass cover slips with cells were transported into a self made basket and washed three times with ice-cold PBS for 5 min. For the incubation with the secondary antibody, which specifically recognizes the origin of the first antibody (Fc-region of mouse or rabbit) and is conjugated with a fluorescent dye, glass cover slips were transferred back into the moist chamber. Secondary antibodies were diluted in PBS in a concentration of 1:300 –1:500, cells were covered with 40 µl of antibody solution and incubated at 37 °C for 45 min. Glass cover slips were transferred to the self-made basket, washed three times (5 min each) with ice-cold PBS and were embedded in a drop of Immu-Mount™ mounting medium. The fluorescent dyes were visualized on a Zeiss 510 invert laser-scanning microscope (optical section: < 0.9 µm; multitrack mode; Alexa-488, λ_{exc}: 488 nm, BP filter: 505 – 530 nm; Cy3, λ_{exc}: 543 nm, BP filter: 560 – 615 nm).