2. METHODS AND MATERIALS

2.1 PCR amplification of the homologous DNA sequences of K$_{Ca}$3.1 gene

The genomic DNA isolated from E14.1 ES cells (a kind gift from Dr. Knobeloch, UKBF, FUB) was used in PCR as the DNA template. The 4.6 kb long homologous DNA sequence, the so called "long arm", was amplified using the Expand long template PCR system (Roche) with 300 ng ES cell DNA, 0.4 µmol/L of each primer

sense: 5'- TTACTCTAGACCCAGATCTGCCGGGCAC- 3',

antisense: 5' – CCCTGTCGACTCTACAGAGTGGGATAGC – 3'.

in the following conditions: 3 min at 94° C for the denaturation then 10 cycles consisting of 30 s at 94° C, 25 s at 58° C and 6 min extension at 68° C, after that, 25 cycles of the same three steps at the same temperature but with additional 15 s prolongation at the extension step per cycle, finally, an extension at 72° C for 10 min.

For the complete knockout targeting construct, the 900 bp short homologous DNA fragment (short arm) named Cssarm was amplified using the High fidelity PCR kit (Roche) in following conditions: 3 min at 94° C for the denaturation, 30 cycles consisting of 30 s at 94° C, 25 s at 58° C and 2 min extension at 72° C, followed by a final extension at 72° C for 10 min. The following primer set was used:

sense: 5'- TGAGTCTAGAAAGCAGACATTTGGAACA- 3'

antisense: 5' –CCTCCTCGAGCCACACTCTTAACACCAAG – 3'

In addition, a test vector was made to optimize the PCR condition for screening the homologous recombination event. The 998 bp homologous sequence called Clsarm used for the test vector construction was a bit longer

than the short arm and it was amplified using following primer set:

sense: 5'- TAAGTCTAGATTCAGACTCTGGTGATAGG- 3'

antisense: 5' –CCTCCTCGAGCCACACTCTTAACACCAAG – 3'.

The PCR condition was the same as that in the amplification of Cssarm.

For the conditional targeting construct, the 852 bp ssarm, the 419 bp sarm45 and the 929 bp lsarm were produced using following primer sets respectively:

sense: 5'- GCTTGCTCGAGTCTGGAAAGCAGACATTTG- 3'

antisense: 5' –TCTGGTCGACTCTTTCAGATGCTTAACC – 3'

sense: 5'- CTTTGGATCCAGATGTTTCTTGGTGTTAAG- 3'

antisense: 5' –CGGCAGATCTGGGTCTTGAGTAAAG – 3'

sense: 5'- GACTCTCGAGATAGGGCTAATCCTAATAAC- 3'

antisense: 5' –TCTGGTCGACTCTTTCAGATGCTTAACC – 3'

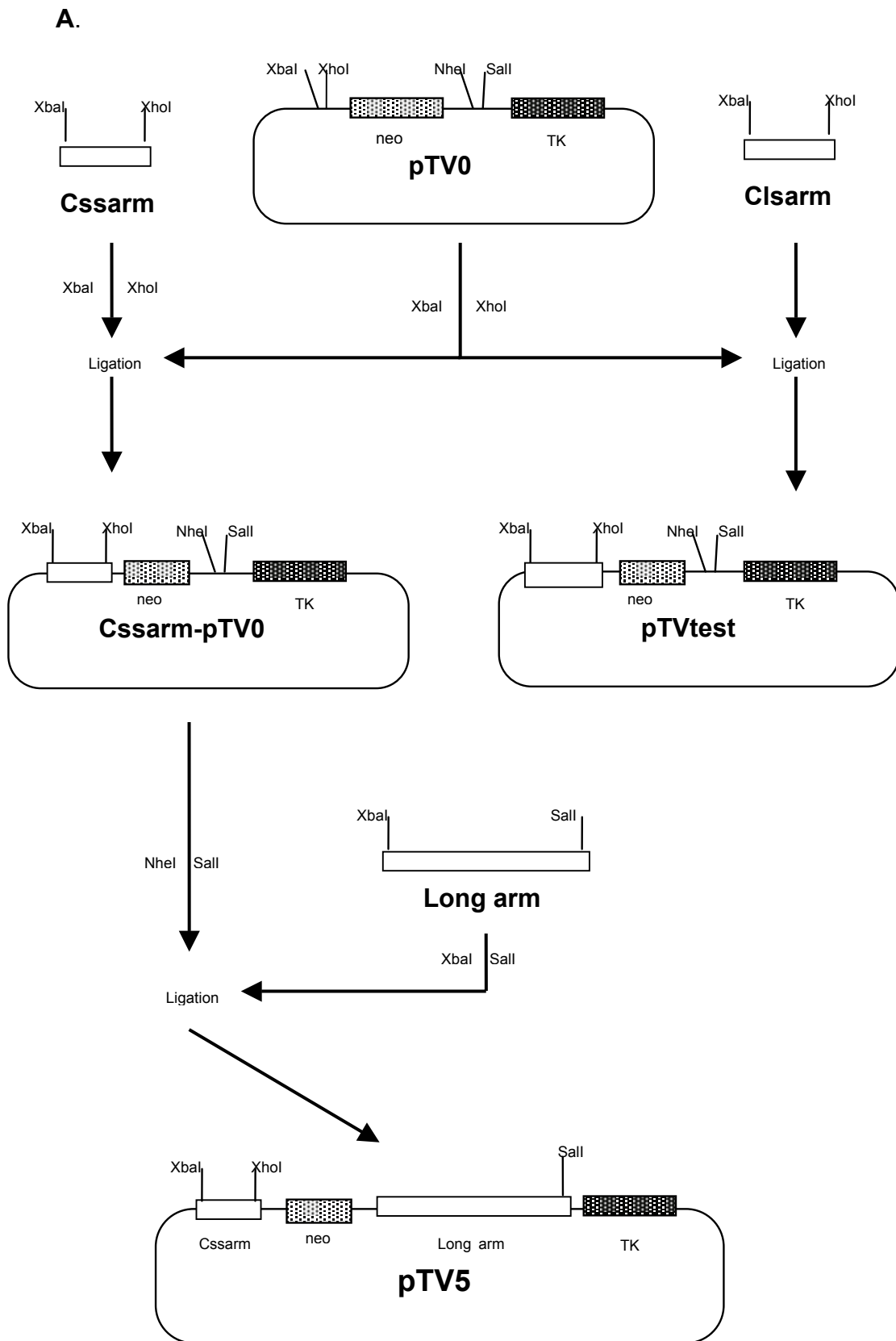The PCR conditions were the same as that in the amplification of Cssarm.

2.2 Targeting vectors for homologous recombination in ES cells

All constructions in this section are outlined in Figure. 2.1.

The complete knockout targeting vector pTV5 was constructed as described below. Firstly, The Cssarm and the pTV0 vector were both digested with two endonucleases XbaI and XhoI to produce the sticky ends for cloning, and then the ligation was performed using the Rapid ligation kit (Roche). Secondly, this construct was digested with NheI and SalI and ligated with the long arm digested with XbaI and SalI. Finally, the two homologous arms were cloned into the pTV0 vector in the same orientation flanking the neo cassette. The TK cassette was downstream of the long arm. The test vector pTVtest was produced by the ligation of Clsarm and pTV0, both of which have cohesive

ends generated by XbaI and XhoI.

For the conditional targeting vector, the Sarm45 was digested with BamHI and BglII and ligated with BamHI digested pFlox vector (provided by Prof. Dr. Bader). The cloning product P1 was treated with XhoI followed by ligation with XhoI and SalI double digested ssarm and lsarm to produce P3 and P2, respectively. P2 will be used as the PCR test vector and P3 was digested with XbaI and SalI followed by ligation with the long arm with the same sticky ends to produce the final targeting construct P4.
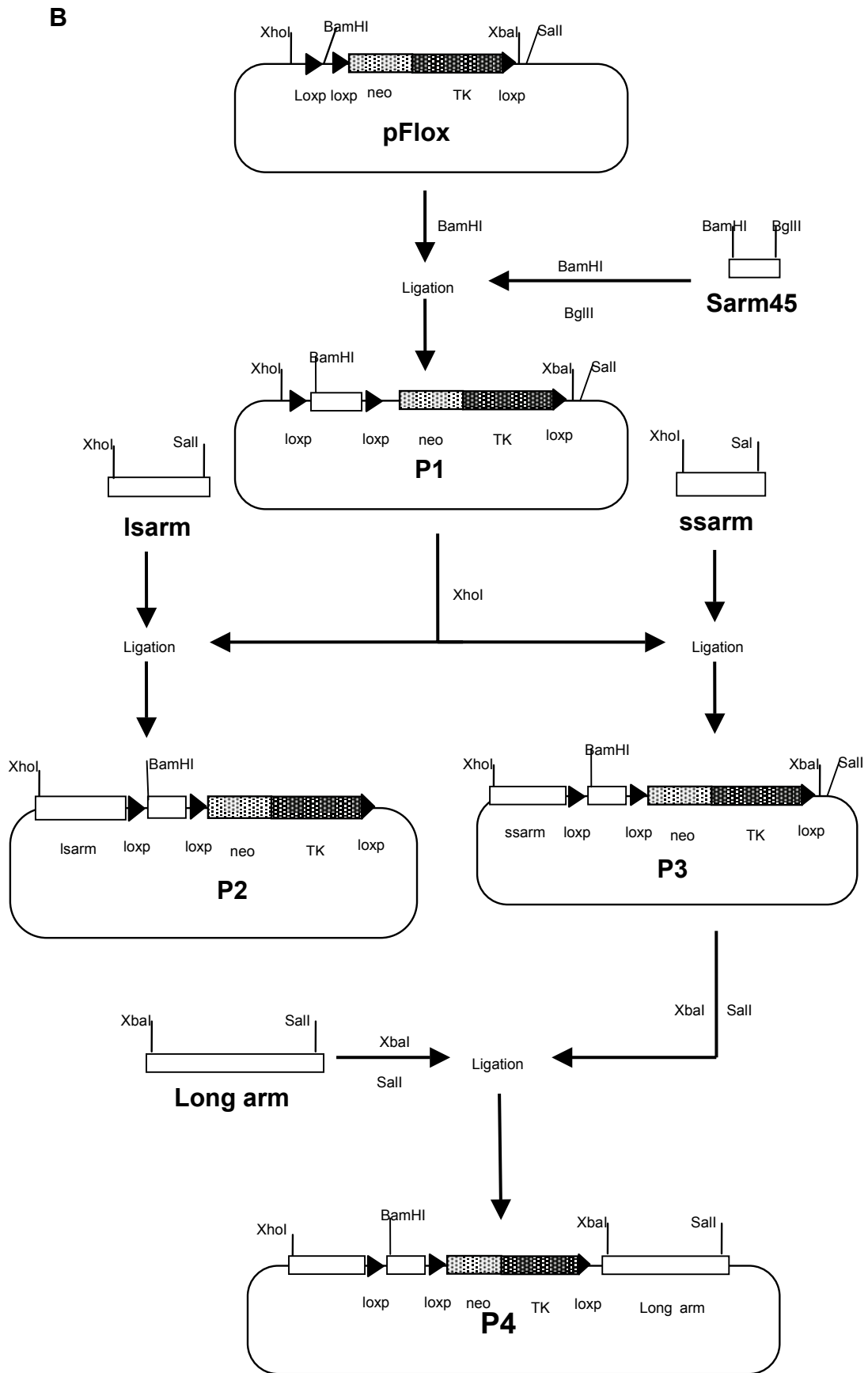
A.

B

Figure 2.1 Cloning of the targeting vectors for homologous recombination. A. Conventional targeting vector cloning. B. Conditional targeting vector cloning. White bars indicate the homologous DNA fragments of the $K_{Ca}3.1$ gene. The neo, TK genes are shown in hatched bars. The black triangles indicate the positions of the loxp sites. The portions of the plasmids represented by the bars are not drawn to scale.

2.3 DNA preparations

2.3.1 Genomic DNA preparations from cultured ES cells for PCR analysis

For the ES cell positive clone screening, cells were picked from the 96-well cell culture plate and centrifuged at 8000 rpm for 10 min, then the supernatant was discarded and 20 µl H_2O was added. For DNA extraction, cells were then incubated in a thermo block at 90°C for 10 min and then spin down. After cooling to RT, 2 µl of a 10mg/ml solution of proteinase K were added and followed by incubation at 55°C for 1 h and at 90°C for 10 min. After that, whole solution was spin down and finally 5 µl was used for PCR as template.

2.3.2 Genomic DNA preparations from mouse tails for PCR analysis

About 3-5 mm of tail was cut from properly marked mouse. Tails were put into 1.5 ml Eppendorf tube and lysed in 90 µl tail digestion buffer containing 100 mmol/L Tris/HCl, 5 mmol/L EDTA (PH 8.0), 0.2% SDS, 200 mmol/L NaCl and 1 mg/ml proteinase K. Samples were incubated in a water bath at 55°C with agitation until lysis was complete (4 to 12 hours). After the digestion tubes were heated at 95°C for 10 min, 500 µl of distilled water was used to dissolve

the DNA.

2.3.3 Genomic DNA preparations from mouse tails for southern analysis

About 0.5 to 1 cm of tail was cut from properly marked mice and was placed into 1.5 ml Eppindorf tube and digested in 0.5 ml tail buffer containing 100 mmol/L Tris/HCl, 5 mmol/L EDTA (PH 8.0), 0.2% SDS, 200 mmol/L NaCl and 1 mg/ml proteinase K, at 55°C with agitation until lysis was complete. The tail should be nicely dissolved so that only hairs and occasional pieces of cartilage and bone are present. The solution may still look dirty due to the dissolved pigments in the tail hair and skin. After the digestion, tubes were centrifuged for 10 min at about 12000 g. Then the supernatant was carefully transferred into a new 1.5 ml tube followed by the addition of 0.5 ml of room-temp isopropanol to each tube and tubes were inverted a few times to produce cottony precipitates. These precipitates were then transferred to a tubes filled with 70% EtOH. Next, the EtOH was removed as much as possible from the precipitates followed by transferring these precipitates into new Eppendorf tubes with 0.2 ml distilled water. Finally these tubes were placed in the 55°C water bath for about 30 min with agitation to dissolve the genomic DNA completely in distilled water.

2.3.4 Small plasmid preparations

Small plasmid DNA preparations of bacterial overnight cultures (2 ml) were done using the Small scale plasmid DNA purification system according to the protocol of the manufacturer (Roche).

2.4 Sequencing of plasmid DNA

DNA sequencing before transfection was performed by SEQLAB GmbH Göttingen, Germany. The output sequences were compared to the published $K_{Ca}3.1$ gene sequence of mouse strain C57BL/6J (gi: 38088123)

2.5 PCR analysis

2.5.1 Detection of homologous recombination with pTV5 targeting vector

In order to detect the positive ES cell clones, PCR reactions were carried out using the following primer set:

Lsarmoldfor: 5'- TAAGTCTAGATTCAGACTCTGGTGATAGG - 3'

Neorev: 5'- CGTGCAATCCATCTTGTTCA - 3'

Lsarmoldfor hybridises to a sequence of the mouse $K_{Ca}3.1$ gene lying outside the short arm homology region of the targeting vector; primer neorev is specific for the neo resistant gene (Fig 2.2). The band indicating homologous replacement of endogenous sequence by the targeting construct is about 1.3 kb in size.

2.5 µl ES cell lysate were added to 22.5 µl amplification mixture, resulting in a final concentration of 1 x PCR reaction buffer, 1.75 mmol/L $MgCl_2$, 400 µmol/L dNTP, 0.4 µmol/L of each primer and 1.25 U *Taq* Polymerase (Invitrogen). The reaction was performed with a thermal cycler (ABI system, USA) with the following temperature profile: a first denaturation step (94°C for 3 min), 35 cycles (35 sec at 94°C, 35 sec at 58°C, and 2 min at 72°C) and a final extension step (10 min at 72°C). The reaction product was analysed on a 1%

agarose gel.

2.5.2 Genotyping of transgenic mice

PCR reactions were carried out for routine mouse genotyping with the following primers:

Kc4: 5'- CTTTGGATCCAGATGTTTCTTGGTGTTAAG - 3'

Exon4rev: 5'- GCCACAGTGTGTCTGTGAGG – 3'

and neorev. Among them Kc4 forward primer binds to the intron which lies upstream of the exon4 of $K_{Ca}3.1$ gene and exon4rev is specific for exon4 (Fig. 2.2), thus the multiplex PCR yielded 320 bp for the neo resistant gene and 160 bp for the wild-type $K_{Ca}3.1$.

To 2.5 µl of tail lysate, 22.5 µl of amplification mixture were added, resulting in a final concentration of 1 x PCR reaction buffer, 1.75 mmol/L $MgCl_2$, 400 µmol/L dNTP, 0.4 µmol/L of each primer and 1.25 U *Taq* Polymerase (Invitrogen). The reaction was carried out in a thermal cycler (ABI system, USA) with the following temperature profile: a first denaturation step (94°C for 3 min), 10 cycles (35 sec at 94°C, 35 sec at 58°C and 50 sec at 72°C) followed by 25 cycles (35 sec at 94°C, 35 sec at 58°C and 50 sec at 72°C with 5 sec extension every cycle) and a final extension step (10 min at 72°C). The reaction product was analysed on a 1% agarose gel.

2.5.3 Detection of homologous recombination with P4 targeting vector

In order to detect the positive ES cell clones after transfection, PCR reactions were carried out using the following primer set:

Neofor: 5'- CGAGTTCTTCTGAGGGGATCA - 3'

Kcnntest: 5'- GCTTTTCCAAATTAGCGGGTA - 3'

The Kcnntest primer hybridises to a sequence lying outside the short arm named ssarm which is homologous to the $K_{Ca}3.1$ gene; the neofor primer is specific for the neo resistant gene (Fig 2.2). The band indicating homologous replacement of endogenous sequence by the targeting construct is about 1.8 kb in size.

To 2.5 µl ES cell lysate, 22.5 µl amplification mixture were added, resulting in a final concentration of 1 x PCR reaction buffer, 2 mmol/L $MgCl_2$, 400 µmol/L dNTP, 0.4 µmol/L of each primer and 1.25 U *Taq* Polymerase (Invitrogen). The reaction was subjected to thermal cycler (ABI system, USA) with the following temperature profile: a first denaturation step (94° C for 3 min), 10 cycles (35 sec at 94°C, 35 sec at 58°C and 2 min at 72°C) followed by 25 cycles (35 sec at 94°C, 35 sec at 58°C and 2 min at 72°C with 10 sec extension every cycle) and a final extension step (10 min at 72°C). The reaction product was analysed on a 1% agarose gel.
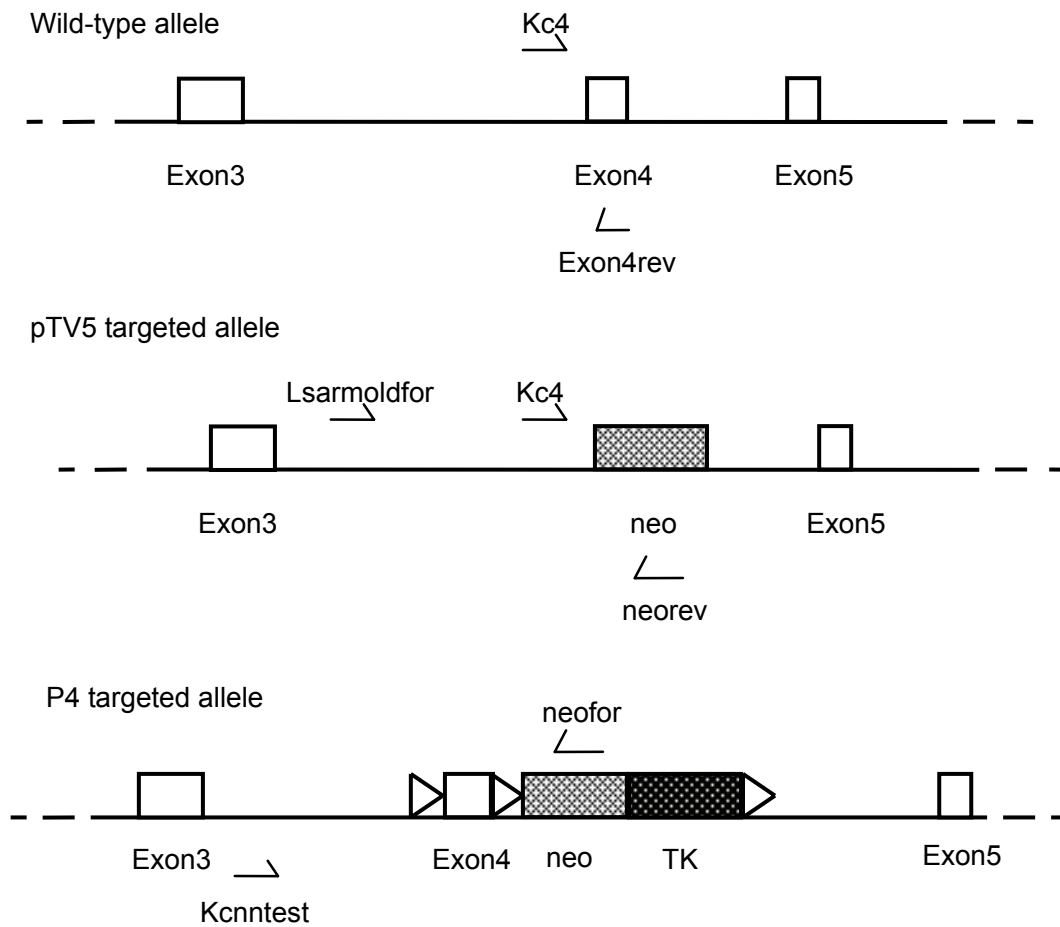
Figure 2.2 Primer positions in PCR analysis. Triangles represent loxp sites.

2.6 Southern analysis

2.6.1 Basic principles

Southern blotting, devised by E. M. Southern in 1975, is a commonly used method for the identification of DNA fragments that are complementary to a known DNA sequence. Southern blotting allows a comparison between the genome of a particular organism and that of an available gene or gene fragment (the probe). It can tell us whether an organism contains a particular

gene, and provide information about the organization and restriction map of that gene. In Southern blotting, chromosomal DNA is isolated from the organism of interest, and digested to completion with a restriction endonuclease enzyme. The restriction fragments are then subjected to electrophoresis on an agarose gel, which separates the fragments on the basis of size. DNA fragments in the gel are denatured (i.e. separated into single strands) using an alkaline solution. The next step is to transfer fragments from the gel onto nitrocellulose filter or nylon membrane. This can be performed by electrotransfer (electrophoresing the DNA out of the gel and onto a nitrocellulose filter), but is more typically performed by simple capillary action. In this system, the gel is placed onto sheet(s) of moist filter paper, a large part of which is immersed in a buffer reservoir. A nitrocellulose membrane is laid over the gel, and a number of filter papers are placed on top of the membrane. By capillary action, buffer moves up through the gel, drawn by the filter paper. It carries the single-stranded DNA with it, and when the DNA reaches the nitrocellulose it binds to it and is immobilized in the same position relative to where it had migrated in the gel. The DNA is bound irreversibly to the membrane by baking at high temperature (nitrocellulose) or cross-linking through exposure to UV light (nylon). The final step is to immerse the membrane in a solution containing the probe - either a DNA (cDNA clone, genomic fragment, and oligonucleotide) or RNA probe can be used. This is DNA hybridization - in other words the target DNA and the probe DNA/RNA form a 'hybrid' because they are complementary sequences and so can bind to each other. The probe is usually radioactively labeled with ^{32}P, often by the synthesis carried out using Klenow fragment of DNA polymerase I which is able to incorporate [^{32}P]dCTP to the probe. Klenow Enzyme catalyzes the addition of mononucleotides from deoxynucleoside-5'-triphosphates to the 3'-hydroxyl terminus of a primer/template DNA. The membrane is washed to

remove non-specifically bound probe, and is then exposed to X-ray film - a process called autoradiography. At positions where the probe is bound, emissions from the probe cause the X-ray film to blacken. This allows the identification of the sizes and the number of fragments of chromosomal genes with strong similarity to the gene or gene fragment used as a probe.
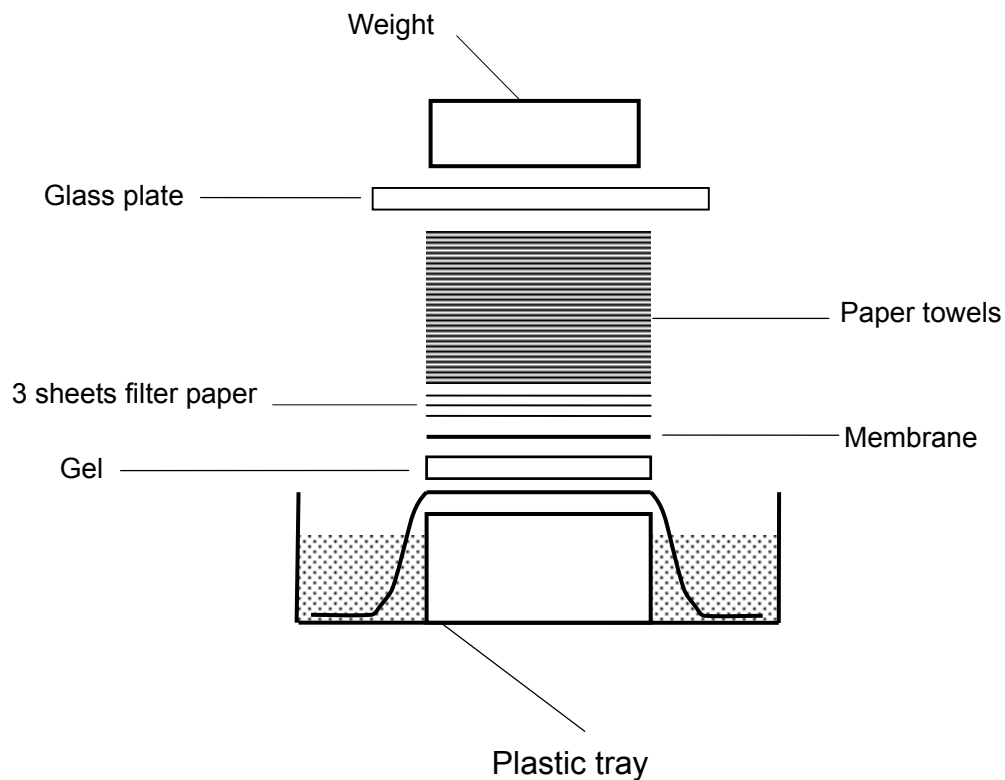

Figure 2.3 Schematic illustration of a capillary blotting apparatus for southern analysis.

2.6.2 Amplification of probes used for the hybridization

The probe used in southern analysis was a 1063 bp DNA fragment amplified using following primer set:

Longsouthfor: 5'- AGATCCTGCTGGAGCTGTTG - 3'

Longsouthrev: 5'- AGGCTGAGGTACTGGGGTGT - 3'

600 ng of mouse genomic DNA was used in PCR as the template and the PCR was carried out with 0.4 µmol/L of each primer, 1.75 mmol/L $MgCl_2$, 400 µmol/L dNTP and 2.5 U *Taq* Polymerase (Invitrogen) in the following conditions: a first denaturation step (94°C for 3 min), 35 cycles (35 sec at 94°C, 35 sec at 58°C, and 2 min at 72°C) and a final extension step (10 min at 72°C). The reaction product was analysed on a 1% agarose gel and purified using Roche PCR product cleaning kit.

2.6.3 DNA gel electrophoresis and blotting

Approximately 10 µg of genomic DNA prepared from each mouse tail were cleaved with 50 U of BglII (Roche) in 30 µl digestion buffer overnight at 37°C. Following the digestion, 10 µl of sample buffer (see below) were added and the whole solution was added to the well of a 0.8% agarose gel (Invitrogen). After successful gel electrophoresis (~80 V for 3-4 h), the DNA samples in the gel were visualized with UV light and photographed. Before blotting, the gel was rinsed in distilled water, and then it was placed in sufficient denaturation buffer (see below) and incubated for 30 min with gentle agitation. After rinsing, the gel was placed in sufficient neutralization buffer (see below) and incubated for 30 min with gentle agitation. The DNA was transferred from the gel to a nylon membrane (Hybond-XL, Amersham Pharmacia Biotech Europe, Germany) using the transfer setup shown in Figure 2.3. The nylon membrane was soaked in 10x SSC (see below) before use. Transfer was done overnight. The next day the membrane with the bound DNA was retrieved from the transfer setup and marked to allow identification of the tracks with a pencil. The DNA was fixed to the membrane by baking at 80°C for 2 hours and kept at room temperature until use.

2.6.4 Hybridization and autoradiography

Twenty minutes of prehybridization in Rapid-hyb buffer (Amersham Pharmacia Biotech Europe, Germany) were followed by two hours' hybridization at 65°C using the same solution supplemented with 1×10^6 cpm/ml of synthesized probe which was ^{32}P labeled using the Rediprime II random prime labeling system (Amersham Biosciences, Europe). Blot was washed once in 50 ml of 2x SSC, 0.1% (w/v) SDS for 20 min at room temperature, then twice in 50 ml of 0.1x SSC, 0.1% (w/v) SDS for 15 min each at 65°C. The membrane was then placed at -80°C in a freezer for autoradiography and the exposure time was 48 hours.

2.6.5 Materials

Chemicals

NaCl, sodium acetate, Tris-(hydroxymethyl)aminomethane (TRIZMA Base, Tris), ethylenediaminetetraacetic acid (EDTA), NaOH, glycerol and sodium dodecylsulphate (SDS) were purchased from Sigma (Taufkirchen, Germany). Sodium citrate was from Calbiochem (Bad Soden, Germany). Bromophenol blue was obtained from SERVA Electrophoresis GmbH (Heidelberg, Germany).

Solutions

Denaturation buffer: 0.5 mol/L NaOH, 1.5 mol/L NaCl. *Neutralization buffer*: 1 mmol/L EDTA, 0.5 mol/L Tris (PH 7.8), 1.5 mol/L NaCl. *20x SSC*: 3 mol/L NaCl, 0.3 mol/L sodium citrate (PH 7.0). *Electrophoresis sample buffer*: 20% glycerol with Bromophenol blue in TE. *TE Buffer*: 10 mmol/L Tris (pH 7.5), 1 mmol/L EDTA.

2.7 RNA analysis

2.7.1 RNA isolation

Total RNA was extracted from mouse tissue or cultured cells using the Roche Total RNA Extraction Kit according to the manufacturer's protocol. In case of extraction from tissue, pretreatment as follows was performed: fresh tissue was immediately frozen in liquid nitrogen and pulverized at -80°C using a mortar and pestle and then the pellet of the pulverized tissue was resuspended in the buffer provided by the kit. After the purification, the RNA was dissolved In RNAase-free water.

2.7.2 RT-PCR

In this study, RT-PCR was employed to test the $K_{Ca}3.1$ gene expression in transgenic mice. Firstly, about 1 µg total RNA extracted from mouse spleen was reverse transcribed in a 50 µl reaction containing 1x RT buffer, 1 ng/µl of random hexamer, 1U/µl of Superscript II™ reverse transcriptase, 2 mmol/L $MgCl_2$, 400 µmol/L dNTP, 4mmol/L of DTT, 1U/µl of RNAase inhibitor (all above from Invitrogen). The reaction was carried out in a thermo cycler (ABI system, USA) with the following temperature profile: 10 min at 25°C, 50 min at 42°C and 15 min at 70°C.

Secondly, 5 µl of cDNA produced in RT was amplified by PCR. In a 50 µl of reaction, 1 x PCR reaction buffer, 1.75 mmol/L $MgCl_2$, 400 µmol/L dNTP, 0.4 µmol/L of each primer and 1.25 U *Taq* Polymerase (Invitrogen) were mixed well. The PCR reaction was performed in a thermal cycler (ABI system, USA) with the following temperature profile: a first denaturation step (94° C for 3 min), 35 cycles (35 sec at 94°C, 35 sec at 58°C and 50 sec at 72°C) and a final

extension step (10 min at 72°C). The reaction product was analysed on a 0.8% agarose gel.

For $K_{Ca}3.1$ cDNA, primer set is:

IK1nestfor: 5' - CCATGCTCCTGCGTCTCTAC - 3'

IK1nestrev: 5' - TACAGCTTGGCCACGAACC – 3'

For the positive control (fibronectin), primer set is:

Fibronestfor: 5'- GGAGAGACTGCGCCCTACT - 3'

Fibronestrev: 5'- GAGCAGGTCAGGAATGTTCA -3'

2.8 Cell isolation and culture

2.8.1 Isolation of endothelial cells

For the isolation of vascular endothelial cells, freshly removed mouse aortas were placed in MEM-Earle medium (Biochrom KG, Berlin, Germany). Vessels were firstly cleaned to get rid of connective tissue then cut open longitudinally to expose the luminal layer. Aortas were incubated in 0.5% trypsine solution (Biochrom KG, Berlin, Germany) at 37°C for 20 - 25 min prior to the isolation of ECs. Using a sharp edge small spoon, the vascular ECs were collected by scraping the luminal side of the open aorta. ECs were then transferred to DMEM medium and cultured at 37°C in a humidified atmosphere with 5% CO_2 until they were attached on glass cover slips suitable for electrophysiological experiments at the same day.

The following culture medium was used:

Dulbecco's modified Eagle's medium (DMEM, Biochrom KG, Berlin, Germany) supplemented with 10% (v/v) fetal bovine serum, 1% sodium pyruvate, 100 U/ml penicillin and 100 µg/ml streptomycin.

2.8.2 Isolation of lymphocytes

For the peripheral lymphocytes isolation, about 1 ml of mouse blood was drawn into a syringe containing 10 U/ml heparin (Roche, Germany) and transferred to a 15 ml falcon tube followed by 1:1 dilution with PBS w/o Ca^{2+}/Mg^{2+} (Biochrom KG, Berlin, Germany). Then this diluted blood was poured slowly and carefully onto 20 ml Ficoll (Amersham Bioscience, Germany) holding in a diagonal manner. The tube was centrifuged at 4°C, 1600 RPM for 30 min. Then the narrow bright interface between serum and Ficoll was collected by a pipette and transferred to a new 15 ml tube followed by washing with PBS w/o Ca^{2+}/Mg^{2+} 3 times and finally plated in culture medium.

In order to isolate untouched $CD4^+$ T-cells from animals, the Dynal® mouse $CD4^+$ negative isolation kit (DYNAL Biotech Inc, Germany) was used. The experiments were performed according to the protocol of the manufacturer. Briefly, mouse spleen lymphocytes or peripheral lymphocytes were prepared and suspended in PBS with 0.1% BSA. These cells, in turn, were mixed well with antibodies against all unwanted cells (e.g. B cells, macrophages/monocytes, natural killer cells, dendritic cells, $CD8^+$ T-cells, granulocytes and erythrocytes) and incubated for 20 min at 4°C. After washing with PBS, cells were incubated with Dynabeads™ for 15 min at 18°C with bidirectional rotation. Bead-captured cells were resuspended by gently pipetting several times and diluted 1:1 with PBS w/0.1% BSA. The tubes with cells inside were placed in the magnet for 2 min and the supernatant containing untouched $CD4^+$ T-cells were transferred to the tissue culture dish with medium.

Freshly isolated peripheral mouse lymphocytes and $CD4^+$ T-cells were kept in

2 cm small tissue culture dish at 37°C in a humidified atmosphere with 5 % CO_2 until use at the same day.

The following culture medium was used:

RPMI 1640 medium (Biochrom KG, Berlin, Germany) supplemented with 10% (v/v) fetal bovine serum, 1% sodium pyruvate, 100 U/ml penicillin and 100 µg/ml streptomycin.

2.8.3 Culture of mouse embryonic fibroblasts

Mitotically inactive mouse embryonic fibroblasts (MEF) were employed as the feeder cells for murine embryonic stem cell culture in this study. These mitotically inactive feeder cells provide support to the ES cells. MEF cells used in this study were isolated from a 13.5 d postcoitus (dpc) embryo derived from a transgenic mouse strain harbouring the neomycin resistant gene.

Cells were grown in 10 cm tissue culture dish at 37°C in a humidified atmosphere with 5 % CO_2 and split usually 1:4 at confluency. In order to make them the feeders for ES cell culture, 10 mg/l mitomycin C (Sigma, Germany) were added to each plate with 5 ml culture media. Dishes were placed at 37°C for 3 h. After incubation, the medium was removed and cells were washed 3 times with PBS. Following trypsinization, cells were collected by rinsing the dish with the medium. Then medium was removed by centrifugation and MEF cells were resuspended and seeded onto gelatin-coated plates to facilitate cell adhesion and in turn for ES cell culture. Gelatin-coated plates were prepared by covering the dish surface with 0.1% gelatin (Gibco) and incubated for at least 30 min. After incubation, excessive gelatins were removed and plates were kept dry for seeding the feeder cells. The feeder cells were used within

one week.

The following culture medium was used:

Dulbecco's modified Eagle's medium (DMEM, Gibco) with 4.5 g/l glucose, supplemented with 10% (v/v) fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin (all Gibco).

2.8.4 Culture of ES cells

The ES cell line described in this thesis was derived from E14.1. This is an embryonic stem (ES) cell line derived from 129/OlaHsd mice (Harlan UK LTD, UK). The parental ES line is called E14TG2a. E14.1 is a subclone of E14TG2a, which was established in Klaus Rajewsky's lab in Cologne, Germany. Embryonic stem cells are totipotent cells with a doubling time of about 12 h. They were seeded at a certain density (about 10 - 20 percent of confluency), as they like to grow in clusters. Therefore, they were monitored twice a day to prevent overgrowth. To avoid temperature shock, ES cell media were always warmed to 37°C before adding to the culture.

One vial of frozen ES cells was thawed by warming up quickly at 37°C then cells were transferred to a tube with 5 ml ES medium. Cells were collected by centrifugation at 300 g and plated onto a 10 cm feeder dish. Cells were grown at 37°C in a humidified atmosphere with 5 % CO_2 and split usually 1:5 at 60% confluency. Culture medium was changed everyday. To freeze one plate of ES cells, cells were suspended in 2.5 ml of medium and 2.5 ml freezing medium (see below) was added slowly and mix gently then aliquoted into five cryopreservation vials. Cells were stored for 2 h at -20°C then transferred to -80°C freezer. Next day they were moved to liquid nitrogen for long-term storage.

Electroporation

The medium of ES cells at about 80% confluency was changed 3 h prior to electroporation. DNA constructs P4 or pTV5 were digested with SalI or NotI, respectively and purified using PCR cleaning kit (Roche, Germany). The final concentration was 20 µg DNA in 40 µl of sterile water. ES cells were collected by trypsinization and transferred to a 15 ml falcon tube and then wash with Cappechi buffer (see below). After washing, 400 µl of Cappechi buffer was added to cells and mixed well. The cell suspension was transferred to a sterile cuvette (BIORAD, Germany). 40 µl DNA was added to this cuvette and any air bubble was avoided. The cuvette, in turn, was placed onto the electroporation machine (Gene Pulser, BIORAD, Germany). The electroporator was set at 240 V with a capacitance of 500 µF. After electroporation, cells were transferred from the electroporation cuvette to 30 ml of ES cell medium and plated onto three 10 cm dishes with feeder cells.

Positive clone selection

The selection was started 1 day after the electroporation. 200 µg/ml G418 (Geneticin, Gibco, Germany) were added into the ES cell medium for 6 days. In addition, cells were exposed to 2 µmol/L gancyclovir (Sigma, Germany) in the last 2 days of G418-selection. When massive cell death appeared from day 3 of selection, debris of dead cells was rinsed off with PBS. One day before the end of selection, 96-well plates coated with mitotically inactive MEF cells were prepared. At the end of double selection, the medium in the dish was replaced with 10 ml PBS. The dish then was placed under the microscope and good-quality colonies were picked based on their morphology and size. In principle, only colonies with irregular three-dimensional growth were picked and colonies that showed any signs of differentiation were not picked. The colonies were picked using a P20 tip mounted on a pipette set at 10 µl and

transferred to wells with 50 µl of trypsin. After picking 12 colonies, colonies were dissociated in trypsin by pipetting 4 - 5 times and 30 µl of cell suspension were transferred to the wells containing feeder cells. The rest of them were transferred to 1 ml eppendorf tubes for genomic DNA extraction for PCR analysis. After picking colonies, the cells in 96-well plates were fed with fresh ES cell medium up to 1 ml and kept at 37°C in a humidified atmosphere with 5 % CO_2.

Expansion of positive ES cell clones

After screening by PCR, positive ES cell clones were identified. These clones were subjected to trypsinization shortly and resuspended in ES cell medium with gentle pipetting to get single cells. ES cells were then plated onto 24-well plates coated with feeder cells. After 2 - 3 days cells were splitted into 6-well plates for microinjection experiment and some cells were frozen down in freezing medium for storage.

The following culture medium was used:

Dulbecco's modified Eagle's medium (DMEM, Gibco) with 4.5 g/l glucose, supplemented with 10% (v/v) fetal calf serum, 200 mmol/L $_L$-Glutamine, 1% nonessential amino acids, 1 x ß-mercaptoethanol (100 x stock: 5 µl ß-mercaptoethanol in 10 ml of PBS), 1000 U/ml LIF, 100 U/ml penicillin and 100 µg/ml streptomycin.

2.8.5 Materials

Chemicals

Dimethyl sulfoxide (DMSO), Hepes, Na_2HPO4, Glucose, ß-mercaptoethanol were from Sigma, Germany. $_L$-Glutamine was from Life Science, Germany.

Leukemia inhibitory factor (LIF) at 10^7 Unit/ml was purchased from Chemicon International, Germany.

Solutions

Freezing medium for ES cells: DMEM supplemented with 80% FCS and 10% DMSO, for other cells: 90% FBS and 10% DMSO. Cappechi buffer: 20 mmol/L Hepes, 137 mmol/L NaCl, 5 mmol/L KCl, 0.7 mmol/L Na_2HPO4, 6 mmol/L Glucose, 0.1 mmol/L ß-mercaptoethanol in PBS and filtrated to be sterile.

2.9 Generation of chimeric mice from transformed embryonic stem cells

Generation and breeding of the $K_{Ca}3.1$-transgenic mouse were under the guidance of a protocol approved by the local Animal Care and Use Committee.

On the day of blastocyst injection, cells in one well of the 6-well plate were washed twice and trypsinized for 2 min at 37°C followed by disaggregation by pipetting up and down about 10 times using a 5 ml pipette so that there was no remaining clumps of cells. Then cells were centrifuged and suspended in 0.5 ml ES cell medium followed by plated into a 24-well plate. Cells were kept at 37°C for 30 min. During that time most of the relatively bigger feeder cells attached to the bottom of the plate whereas ES cells suspended yet in medium were transferred to eppendorf tubes (about 250 µl each) for microinjection. Cultured and transformed ES cells were injected into C57Bl/6 blastocysts and reimplanted into pseudo-pregnant foster mice by Ms. Becker in TCF, MDC, Berlin-Buch. Chimerism of the offspring was estimated by the proportion of agouti coat colour. Chimeric mice were mated to wild type

C57Bl/6 partners (Charles River, Germany), and brown offspring were screened for the presence of the mutant allele using a PCR assay and verified with a Southern blot analysis.

2.10 Mice husbandry and breeding

All the transgenic animals used in this study were housed in the animal facility in BMFZ, University of Marburg, Germany. Animals were housed at most 6 mice per cage in a temperature-controlled room (22 ± 2°C) with a 12-h light/dark cycle and fed standard chow (0.25% sodium, SNIFF Spezialitäten, GmbH, Soest, Germany) with free access to water. The heterozygous offspring from chimeras were interbred in a harem system in which one male heterozygous is housed with two or three heterozygous females. Female mice were separated before delivery. Pups were weaned no later than 23 days after birth and separated based on their sex. The birth date and pedigree of each breeding offspring were recorded on the cage card. Pups were ear tagged and the tail biopsies were obtained when they were two weeks old. After genotyping, the homozygous mutant mice and their wild type littermates were used in our study.

2.11 Patch-clamp experiments

2.11.1 Basic principles

Broadly speaking, electrophysiological techniques to record ion fluxes across a membrane can be divided into indirect methods that employ extracellular

electrodes, as in many non-invasive methods such as electroencephalo/cardio/myo-grams, and direct methods that utilize micropipettes to make contact with the cell of interest. The latter include intracellular recording techniques, where the pipette penetrates the cell, and patch clamp, where the pipette makes contact with the cell but does not penetrate. In the present work, the patch-clamp technique was applied to examine the $[Ca^{2+}]_i$-activated potassium currents through the corresponding membrane channel protein. The patch-clamp technique was developed by Erwin Neher and Bert Sakmann (Neher & Sakmann, 1976; Hamill *et al*., 1981) and is widely used to record whole cell currents from small cells as well as to measure the activity of single ion channels (Sakmann & Neher, 1983; Ogden & Stanfield, 1994; Numberger & Draguhn, 1996). Moreover, this technique allows manipulating easily the fluid on the extracellular or the intracellular side of the membrane during a recording.

In order to enable experimenters to do those various studies mentioned above, patch-clamp technique will be used in different configurations.

Cell-attached patch mode

The simplest patch-clamp configuration (in terms of physical manipulation) is cell-attached patch mode. Every patch-clamp experiment starts with this situation. The micropipette (tip diameter ~ 1 µm) is positioned against the cell membrane where the glass makes a very strong connection, resulting in a tight (high resistance, above 1GΩ) seal (figure 2.6). Ion channel activity in the tiny patch of membrane surrounded by the tip of the pipette can now be studied. The cell-attached patch mode is therefore a single-channel configuration. This configuration leaves the cell intact, and is therefore the most frequently used configuration to study single channels and the simplest to obtain. However, it does not allow easy manipulation of the media on either

side of the membrane, and the membrane potential cannot be directly measured.

Whole cell mode

If the patch of membrane under the pipette tip in cell-attached patch mode is ruptured by applying suction to the pipette (Figure 2.6), then the pipette solution and the electrode make direct electrical contact with the cytoplasm. In this configuration, the patch electrode, electrically, is on one side of the plasma membrane and the ground electrode is on the other, therefore the membrane potential can be recorded directly. A patch pipette tip is sufficiently wide to allow the dialysis of the cell by the pipette-filling solution. Because the volume of the cell is negligible compared with that of the patch pipette, the composition of the intracellular fluid can be considered equal to that of the pipette-filling solution. However, unknown cytosolic factors relevant to the subject of study can be unwittingly washed out. To avoid this, it is now possible to apply perforated patch clamp, where electrical contact with the cytosol is established by adding a membrane-perforating agent to the pipette solution. The agent (nystatin or amphoteracin B) perforates the membrane so that only small molecules such as ions can pass through, leaving the cytoplasm's organic composition largely intact.

Inside-out excised patch configuration

The inside-out excised patch is obtained from a cell-attached patch configuration, where the pipette is pulled away, leaving a patch with the cytosolic side facing the bath (Figure 2.6). Inside-out patches are ideal for studying the effects of cytosolic factors on the isolated channel. Since cytosolic components may be lost in the inside-out configuration, the activity of the ion channels may differ.

Outside-out excised patch configuration

The outside-out patch is obtained by simply pulling away the patch pipette from a whole-cell configuration. The membrane will eventually break and, owing to the properties of the phospholipids, fold back on itself into a patch covering the pipette (Figure 2.6). Outside-out patches can be used to study the effects of extracellular factors on the channels, because the bath composition can be altered easily during recording. Clearly, the disadvantage of this configuration is the same as that in the whole-cell mode: some potential intracellular modulators of the ion channel of interest can be washed out entirely by the pipette solution.
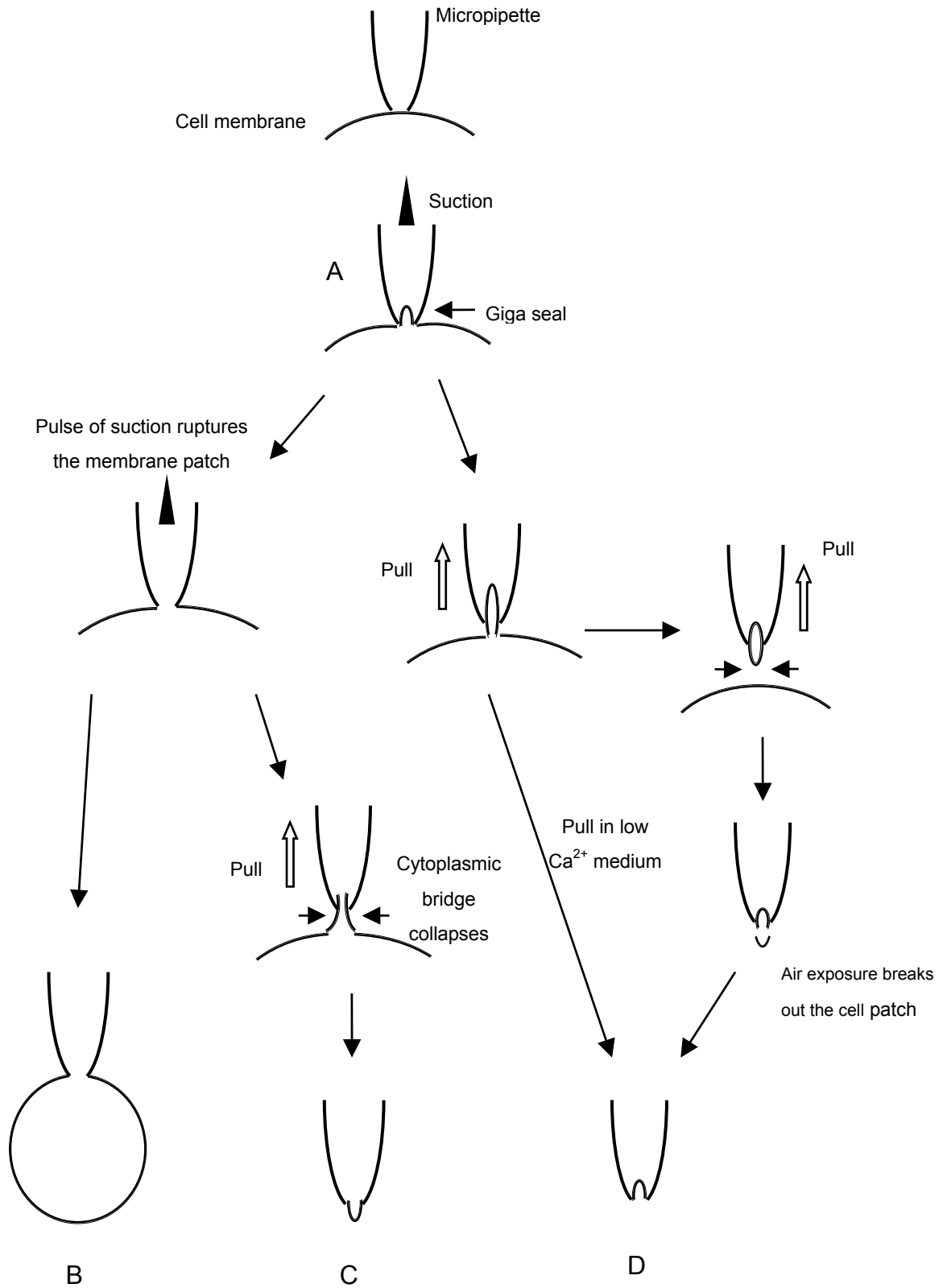
Micropipette

Cell membrane

Suction

A

Giga seal

Pulse of suction ruptures
the membrane patch

Pull

Pull

Pull

Pull in low
Ca^{2+} medium

Cytoplasmic
bridge
collapses

Air exposure breaks
out the cell patch

B

C

D

Figure 2.6 Schematic illustrations of the four different modes of patch clamp: **A**. Cell-attached mode; **B**. whole-cell mode; **C**. outside-out configuration; **D**. inside/out configuration.

2.11.2 Recording conditions for patch-clamp experiments

For the electrophysiological recordings, cells were freshly isolated from animal, and then placed in a recording chamber with 1 ml of normal saline solution as the bath solution (137 mmol/L NaCl, 4.5 mmol/L Na_2HPO_4, 3 mmol/L KCl, 1.5 mmol/L KH_2PO_4, 0.4 mmol/L $MgCl_2$, 0.7 mmol/L $CaCl_2$ at pH7.4). The chamber was linked to a perfusion system for exchanging the bath solution by air pressure. All experiments were performed at room temperature (~22°C). An inverted Axiovert microscope (Zeiss, Germany) was employed to visualize cells and for the micropipette to move precisely to form the tight seal with the cell membrane, a micromanipulator connected with a small pressure system was used. All these equipments were placed on an anti-vibration table. Membrane currents were recorded with a HEKA electronics (Lambrecht, Germany) EPC-9 patch-clamp amplifier. Patch pipettes were prepared from borosilicate glass with a tip resistance of 3 to 5 MΩ. Seal resistance ranged from 4 to 10 GΩ.

The patch pipette was filled with a KCl solution containing: 135 mmol/L KCl, 1 mmol/L $MgCl_2$, 1 mmol/L EGTA, 0.955 mmol/L $CaCl_2$, 1 mmol/L Na_2ATP, and 5 mmol/L Hepes, adjusted to pH 7.2 with KOH (corresponding to 3 μmol/L $[Ca^{2+}]_i$).

For whole-cell recordings, current-voltage (*I-V*) relations were routinely obtained every 5 s by applying voltage ramps from -120 to 100 mV with duration of 500 ms. Data were low-pass-filtered (23 dB, 1000Hz) at a sample time of 0.5 ms.

2.11.3 Data analysis

For calculation of mean current densities, current amplitudes were standardized to the cell capacitance. Leak currents were subtracted before data acquisition by using the pulse data acquisition software. To calculate the median effective concentration (EC_{50}) of cytosolic free Ca^{2+}, Boltzmann equation was used: $f(x)=(A_1-A_2)/(1+e^{(x-x0)/dx})+A_2$, where A1 is the maximal activation; A_2, the minimal activation; x_0, $EC_{50;}$ dx, the slope of the curve.

2.11.4 Materials

Charybdotoxin, Apamin, DC-EBIO, UCL1684 were purchased from Sigma, Germany. TRAM-34 was a kind gift from Dr. Wulff (UC, Davis). Other general chemicals were all from Sigma, Germany.

2.12 Blood pressure measurements

To investigate whether the $K_{Ca}3.1$-deficient mice have abnormal blood pressure, two methods, namely the tail-cuff plethysmography and telemetry, were applied to $K_{Ca}3.1$ knockout and wild-type littermates.

2.12.1 Tail-cuff experiments

12 to16-week old mice were treated with Dormicon® (1mg/kg) (Roche, Germany) and kept on a warm plate for experiments. The mouse pulse and tail arterial pressure were detected and transferred to the NIBP controller (model no: ML125). Together with a data recording system (model no: ML840)

the pressure and pulse signal were digitalized, displayed and finally recorded in the computer. Data were evaluated with the Chart software. All of equipments mentioned above were purchased from ADInstruments GmbH, Spechbach, Germany.

2.12.2 Telemetry experiments

In this study the telemetry experiments were performed in collaboration with Dr. V. Gross at the MDC, Berlin, Germany. Briefly, before implantation of a TA11PA-C20 blood pressure device (Data Sciences International, St. Paul, MN), the zero offset was measured, and the unit was soaked in 0.9% NaCl. Mice were anesthetized with isoflurane (CuraMed Pharma, Karlsruhe, Germany). The pressure sensing catheter was advanced via the right femoral artery into the abdominal aorta and the transmitter was placed in a subcutaneous pocket along the right flank. During surgery and in the recovery period the mice were placed on a heated table to maintain the body temperature. All mice were allowed 7 day's recovery from surgery before baseline blood pressure. The mice got normal chow and water.

The data from the TA11PA-C20 device were transmitted via radio frequency signals to a receiver below the cage and thereafter collected using the Dataquest ART system, version 2.1 (Data Sciences International), which allowed us to detect, collect and analyze signals from several animals simultaneously. The data were sampled every 5 min for 10 s continuously day and night with a sampling rate of 1000 Hz and stored on a hard disk. Systolic, diastolic pressures as well as heart rate were recorded using the Dataquest software 2.1. The heart rate was computed from the pulse intervals of the blood pressure recordings. For statistical analysis 3 days of baseline values were used.

2.13 Pressure myography

Carotid arteries (CA) were freshly dissected from $K_{Ca}3.1$-deficient mice and their wild-type littermates and segments of 2 to 2.5 cm in length were cannulated with micropipettes in an experimental chamber mounted on the stage of a Zeiss Axiovert 100 microscope. Vessel diameter was continuously monitored with a video camera. The bath and perfusion solution contained (in mmol/L) 145 NaCl, 1.2 NaH_2PO_4, 4.7 KCl, 1.2 $MgSO_4$, 2.0 $CaCl_2$, 5.0 glucose, 2.0 pyruvate, and 3.0 3-[N-morpholino] propane sulfonic acid (MOPS) along with 1 g/100ml bovine serum albumin (BSA) adjusted to pH 7.4 at 37°C. Carotid arteries were pressurized to 80 mmHg with a pressure myograph system (J.P. Trading P100, Aarhus, Denmark) and continuously perfused at a flow rate of 0.2 ml/min. To block NO and prostacyclin synthesis, the NO-synthase inhibitor N^G-nitro-L-arginine (L-NNA, 100 µmol/L) and the cyclooxygenase inhibitor indomethacin (10 µmol/L) were added to both the bath and perfusion solution. After an initial equilibration period of 40 min, carotid arteries were preconstricted with 1 µmol/L phenylephrine in the bath solution. After development of a stable tone, the endothelium-derived hyperpolarizing factor (EDHF)-mediated vasodilatory responses, which were independent on NO and prostacyclin, were determined by perfusion with increasing concentrations of acetylcholine (ACh, 1 nmol/L to 10 µmol/L) alone or with 10 µmol/L DC-EBIO. Diameter changes were expressed as a percentage of the maximal dilation measured in response to 1 µmol/L sodium nitroprusside (SNP). All chemicals were from Sigma, Germany, except that DC-EBIO was purchased from Tocris, Germany.

2.14 Histological studies

Histological studies were performed in cooperation with Prof. Dr. Moll at the department of Neuropathology, University of Marburg.

In brief, animal organs were freshly removed and quickly placed in neutral-buffered formalin (10%) solution. After fixation, specimens were embedded in paraffin and then cut into about 8 µm thick sections. The combination of hematoxylin and eosin were used to stain the sections. All chemicals were purchased from Sigma, Germany.

2.15 Statistical analysis

If not otherwise stated, data are given as mean±SE and comparisons between groups were made with unpaired t-tests. Values of $P<0.05$ were considered statistically significant.