

1. INTRODUCTION

The plasma membrane acts as a barrier between the cytoplasm and the extracellular space in all living cells. The flow of ions across the plasma membrane is assisted by various membrane proteins such as ion channels and transporters. Ion channels are membrane-spanning proteins containing a hydrophilic pore through which ions flow down their electrochemical gradient. The existence of ion channels was hypothesized by the British biophysicists Alan Hodgkin (Hodgkin 1964) and Andrew Huxley (Huxley 1964) as part of their Nobel Prize-winning theory of the nerve impulse, published in 1952 and strongly confirmed by patch-clamp technique invented in 1970s by Erwin Neher and Bert Sakmann (Neher and Sakmann 1976).

1.1 Potassium channels

The superfamily of mammalian potassium channels comprises around 300 different members (Hille, Armstrong *et al.* 1999). Potassium (K^+) channels are highly selective for potassium ions over other cations, for instance, Na^+ and Ca^{2+} . The GYG motif in the pore is responsible for this K^+ selectivity (Doyle DA, Cabral JM *et al.* 1998; Roux 2005). K^+ channels are multimeric membrane-spanning proteins. Apart from the selectivity filter in the central pore, K^+ channels vary in membrane topology, subunit composition and mechanisms of activation and inactivation (Yellen 2002). Figure 1.1 depicts the topologies of the three major types of K^+ -channel subunits i.e. of voltage-gated and/or Ca^{2+} -sensitive K^+ channel with six transmembrane segments and a

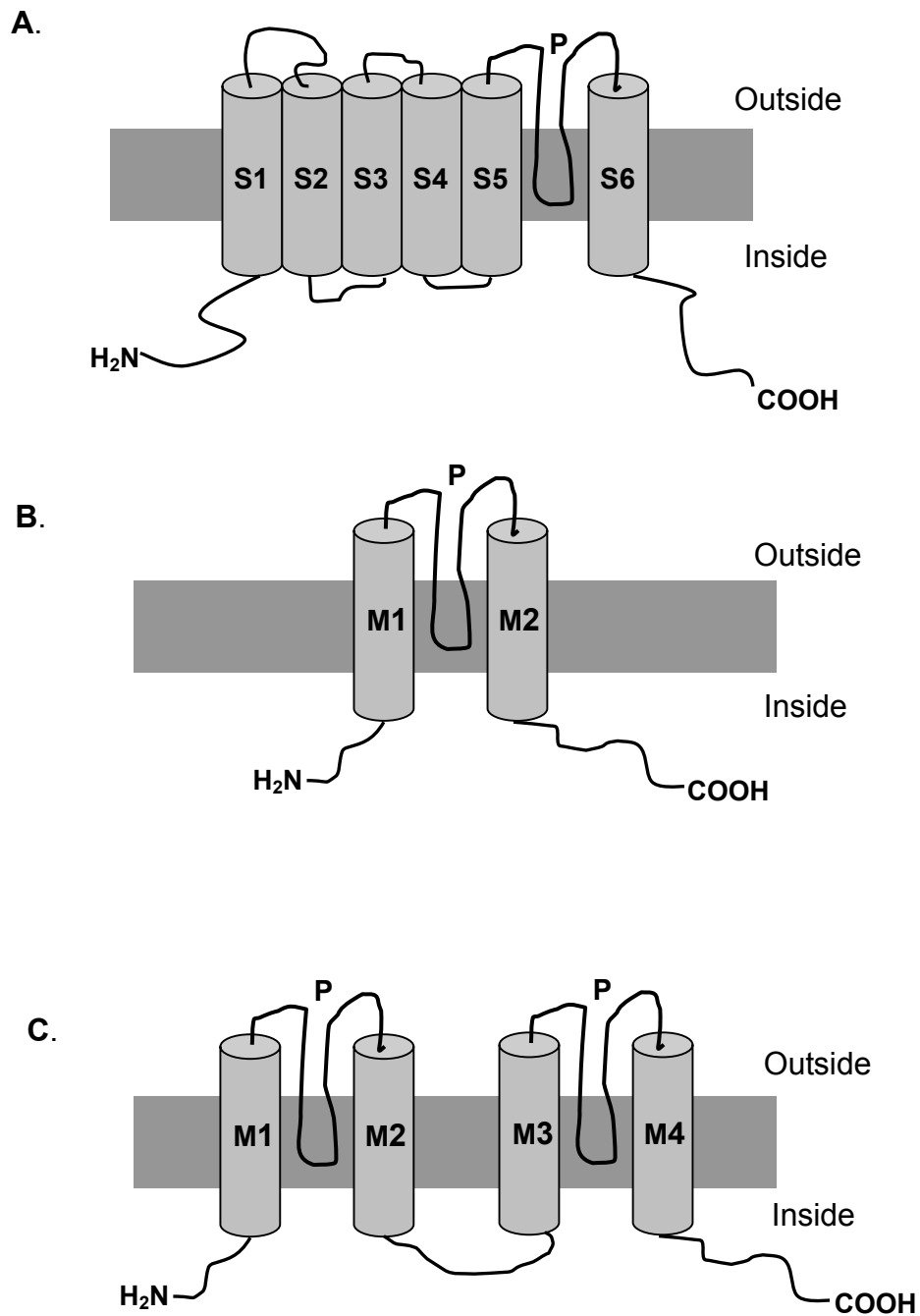


Figure 1.1 Schematic representation of the putative membrane folding of K channel subunits. **A.** K_V and K_{Ca} channel subunits contain six transmembrane segments (S1–S6) and one pore region (P). **B.** The K_{ir} channel subunit contains two membrane-spanning regions and one pore. **C.** The K_{2p} channel subunit contains four membrane-spanning regions and two pores.

single pore (Figure 1.1 A), inwardly rectifying K^+ channel with two transmembrane segments and a single pore (Figure 1.1 B) and two-pore K^+ channel with four transmembrane segments (Figure 1.1 C).

1.2 Ca^{2+} -sensitive K^+ channels

A major family of potassium channels with six transmembrane segments and a single pore is formed by five subfamilies comprising the Ca^{2+} -sensitive K^+ channels. They are named as $K_{Ca}1.1$, $K_{Ca}2.1$, $K_{Ca}2.2$, $K_{Ca}2.3$, and $K_{Ca}3.1$ according to the new nomenclature (Gutman, Chandy *et al.* 2003). All these K_{Ca} channels are formed by tetramers of pore-forming subunits (Figure 1.1 A). K_{Ca} channels between subfamilies differ in their single-channel conductance, subunit composition, mode of Ca^{2+} -activation, voltage-dependence, and pharmacology. K_{Ca} channels have been found in a variety of tissue and cells including nerve, muscle, endothelium, pituitary or chromaffin cells in both vertebrates and invertebrates (Latorre, Oberhauser *et al.* 1989; Vergara, Latorre *et al.* 1998).

$K_{Ca}1.1$ channel, previously named BK, maxi K or Slo channel, is a ubiquitous large conductance K_{Ca} channel, with a single channel conductance in the range of 200-300 pS (Adelman, Shen *et al.* 1992; Atkinson, Robertson *et al.* 1991; Butler, Tsunoda *et al.* 1993). It was found that the protein complex of $K_{Ca}1.1$ channel consists of four pore-forming subunits with a large carboxyl terminal tail domain that plays an important role in calcium sensing by forming a “calcium bowl” for Ca^{2+} binding and protein-protein interactions with several channel modulators (Schopperle, Holmqvist *et al.* 1998; Schreiber and Salkoff 1997; Wei, Solaro *et al.* 1994; Xia, Hirschberg *et al.* 1998). $K_{Ca}1.1$ channel is often

associated with an auxiliary subunit (β subunit) in native tissue and the auxiliary β subunit increases Ca^{2+} -sensitivity (Dworetzky, Boissard *et al.* 1996; Garcia-Calvo, Knaus *et al.* 1994; Knaus, Folander *et al.* 1994; McManus, Helms *et al.* 1995; Tseng-Crank, Godinot *et al.* 1996). Under physiological conditions, $\text{K}_{\text{Ca}1.1}$ channel gating is not only regulated by free intracellular calcium, but also by membrane voltage, i.e. channel open probability increases with membrane depolarization. The voltage-sensor is located in S4 transmembrane domain containing seven positively charged amino acids (Barrett, Magleby *et al.* 1982; Cox, Cui *et al.* 1997; Horrigan and Aldrich 1999; Horrigan, Cui *et al.* 1999; Talukder and Aldrich 2000). The activity of $\text{K}_{\text{Ca}1.1}$ channel can also be modulated by a wide variety of substances such as channel blockers e.g. the scorpion toxins, iberiotoxin and charybdotoxin, and channel openers e.g. NS1619 as well as endogenous second messengers in addition to Ca^{2+} such as cGMP and cAMP (Archer, Huang *et al.* 1994; Biedermann, Skatchkov *et al.* 1998; Bolotina, Najibi *et al.* 1994; Carrier, Fuchs *et al.* 1997; Drouin and Hermann 1994; Kaide, Zhang *et al.* 2001; Lang, Harvey *et al.* 2000; Lu, Mazet *et al.* 1998; Mistry and Garland 1998; Nara, Dhulipala *et al.* 2000; Robertson, Schubert *et al.* 1993; Shin, Chung *et al.* 1997; Wang, Wu *et al.* 1997).

The $\text{K}_{\text{Ca}2.X}$ subfamily of small-conductance K_{Ca} consists of three members i.e. $\text{K}_{\text{Ca}2.1}$, $\text{K}_{\text{Ca}2.2}$ and $\text{K}_{\text{Ca}2.3}$ (Gutman, Chandy *et al.* 2003). Besides the much lower single-channel conductance of approx. 10 pS, $\text{K}_{\text{Ca}2.X}$ channels also differ from $\text{K}_{\text{Ca}1.1}$ by being non-voltage regulated. This is due to the fact that $\text{K}_{\text{Ca}2.X}$ channels have only two positively charged amino acids in the S4 segment (Kohler, Hirschberg *et al.* 1996). Unlike $\text{K}_{\text{Ca}1.1}$, $\text{K}_{\text{Ca}2.X}$ channels show weak inward-rectification due to a reduction of current amplitudes at positive membrane potentials. This type of rectification is caused by intracellular

divalent cations like Mg^{2+} (Soh and Park 2001; Soh and Park 2002). The mechanism of Ca^{2+} activation of $K_{Ca2.X}$ is different from that of $K_{Ca1.1}$. While $K_{Ca1.1}$ has direct Ca^{2+} binding site (“ Ca^{2+} -bowl”) at the C-terminus, the Ca^{2+} sensitivity of the $K_{Ca2.X}$ attributes to constitutively bound calmodulin. Binding of Ca^{2+} to calmodulin leads to conformational changes and thus channel opening. The EC_{50} for $K_{Ca2.X}$ Ca^{2+} activation ranges within 400-800 nmol/L and is therefore much lower than that for $K_{Ca1.1}$ (1-10 μ mol/L) (Fanger, Ghanshani *et al.* 1999; Keen, Khawaled *et al.* 1999; Xia, Fakler *et al.* 1998; Zhang, Kohli *et al.* 2001). The X-ray crystallography and biochemical studies showed that the Ca^{2+} induced activation of $K_{Ca2.X}$ channels occurs via the N-terminal lobe of CaM (Keen, Khawaled *et al.* 1999; Schumacher, Rivard *et al.* 2001). The CaM binding domain in $K_{Ca2.X}$ subunits consists of about 90 amino acids and is located in the C-terminal cytosolic part close to the S6 transmembrane segment (Keen, Khawaled *et al.* 1999; Xia, Fakler *et al.* 1998). Recent studies demonstrated that CaM is not only essential for the $K_{Ca2.X}$ channel gating, but is also required for assembly and trafficking (Lee, Ngo-Anh *et al.* 2003). A $K_{Ca2.2}$ molecule with a mutation in CaM binding domain showed disrupted membrane targeting and a reduced affinity for CaM (Kolski-Andreaco, Tomita *et al.* 2004; Tomita, Shakkottai *et al.* 2003). Based on the molecular biological and immunohistochemical studies, expression of $K_{Ca2.X}$ channels was found mainly in the nervous system (Hosseini, Benton *et al.* 2001; Kohler, Hirschberg *et al.* 1996; Stocker 2000; Tacconi, Carletti *et al.* 2001). In CNS neurons, the activation of $K_{Ca2.X}$ channels is believed to contribute to the slow afterhyperpolarization (AHP) following single or multiple action potentials (Bond, Herson *et al.* 2004; Stocker, Hirzel *et al.* 2004; Vogalis, Storm *et al.* 2003). Other studies in $K_{Ca2.3}$ transgenic mice showed that overexpression of $K_{Ca2.3}$ channel in mice produced abnormal breathing patterns during hypoxic challenge and compromised parturition (Bond, Sprengel *et al.* 2000). Moreover,

it has been shown that $K_{Ca2.3}$ is the only $K_{Ca2.X}$ channel expressed in mice ECs and the modulation of the expression level of $K_{Ca2.3}$ channel in endothelium can influence cardiovascular functions and that suppression of channel expression increases systolic blood pressure (Mark S. Taylor, Adrian D. Bonev *et al.* 2003).

$K_{Ca3.1}$, the only member of the $K_{Ca3.X}$ subfamily, is similar to $K_{Ca2.X}$ channels in terms of voltage-independence, inward rectification, although the single-channel conductance of 35 pS is considerably higher than that of $K_{Ca2.X}$. Moreover, channel pharmacology is different. For instance, $K_{Ca3.1}$ is not blocked by apamin but by charybdotoxin (Eichler, Wibawa *et al.* 2003; Kohler, Brakemeier *et al.* 2001; Kohler, Degenhardt *et al.* 2000; Kohler, Wulff *et al.* 2003), which is also known to block $K_{Ca1.1}$. $K_{Ca3.1}$ is also blocked by the antifungal clotrimazole and its more selective derivatives TRAM-34 (Grgic, Eichler *et al.* 2005; Kohler, Eichler *et al.* 2005; Wulff, Gutman *et al.* 2001; Wulff, Knaus *et al.* 2004; Wulff, Miller *et al.* 2000). This channel is expressed in several tissues and importantly in the endothelium (Eichler, Wibawa *et al.* 2003; Ishii, Silvia *et al.* 1997; Joiner, Wang *et al.* 1997; Kohler, Degenhardt *et al.* 2000). As $K_{Ca3.1}$ is the main focus of this thesis, channel properties and its functional roles in different tissues and organs are introduced in more details in the following sections.

1.3 K_{Ca}3.1 channel gene and biophysics

The K_{Ca}3.1 channel (intermediate-conductance Ca²⁺-activated K⁺ channel), previously named SK4, IK_{Ca}1, was first characterized in erythrocytes, where the channel mediates membrane hyperpolarization and cell volume (Gardos 1958). The gene encoding K_{Ca}3.1 was identified in 1997 by three independent groups (Ishii, Silvia *et al.* 1997; Joiner, Wang *et al.* 1997; Logsdon, Kang *et al.* 1997). The K_{Ca}3.1 gene consists of nine exons (Figure 1.2). Figure 1.2 also depicts the localization of the exon-intron borders within the coding region, and the localization in the human, rat and mouse genomes. Structurally, K_{Ca}3.1 is closest to the K_{Ca}2.X channel subfamily, showing about 40% of identity in the cDNA sequence (Ghanshani, Wulff *et al.* 2000; Ishii, Silvia *et al.* 1997; Logsdon, Kang *et al.* 1997).

Similar to other K_{Ca} channels, K_{Ca}3.1 is activated by small increase of intracellular Ca²⁺ ($\approx 0.2 \mu\text{mol/L}$) above resting levels. At about $0.3 \mu\text{mol/L}$ free Ca²⁺ the channel is half maximally activated (Ishii, Silvia *et al.* 1997). The K_{Ca}3.1 has a mean single channel conductance of about 35 pS (Grygorczyk and Schwarz. 1983; Ishii, Silvia *et al.* 1997; Jensen, Dorte Strøbæk *et al.* 1998; Logsdon, Kang *et al.* 1997). Figure 1.3 shows single channel traces recorded from human endothelial cells and the Ca²⁺ dependence of the channel activation (Kohler, Degenhardt *et al.* 2000).

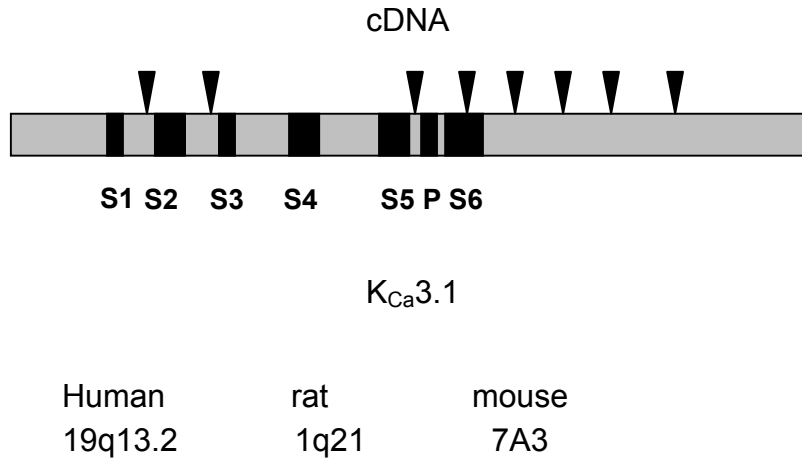


Figure 1.2 Organization of the gene coding for the K_{Ca}3.1 channel. The open gray bar represents the cDNA sequence, and dark bands stand for the six transmembrane segments S1-S6 and the pore region (P). The black arrowheads indicate the exon junctions (introns are not shown in this coding region). The chromosomal location of the gene is given for the human, rat and mouse genome.

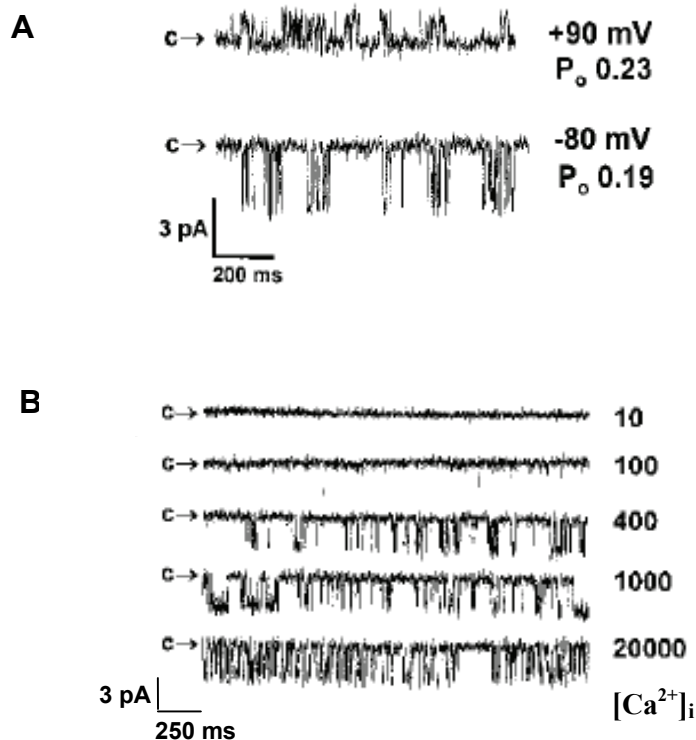


Figure 1.3 Single channel properties of hK_{Ca}3.1. P_o indicates channel open probability. **A.** Single channel inward (down) and outward (up) current recorded from a inside-out patch using symmetric K⁺ solution and 400 nmol/L free Ca²⁺. **B.** Representative single channel traces recorded at different cytosolic free Ca²⁺ concentration (from Kohler *et al.* 2000). Membrane potential was -80 mV. c indicates the close state.

1.4 K_{Ca}3.1 channel gating

Similar to K_{Ca}2.X channels and unlike K_{Ca}1.1, Ca²⁺ sensitivity of the K_{Ca}3.1 channel is conferred by the intimate interaction of calmodulin (CaM) with each subunit (Fanger, Ghanshani *et al.* 1999). In their study, Fanger *et al.* found that CaM is constitutively bound to the C-terminal of K_{Ca}3.1 channel in the absence of Ca²⁺. Moreover, one CaM molecule binds to one subunit of the channel protein, and the concerted action of all four molecules is necessary for the channel gating. An almost identical finding was reported by Xia and colleagues for K_{Ca}2.X channels (Xia, Fakler *et al.* 1998). This may be due to the high similarity of CaM binding sequence on the C-tail between the K_{Ca}2.X and K_{Ca}3.1 channels. Further studies on crystal structures of other potassium channels (Doyle DA, Cabral JM *et al.* 1998; Holmgren, Shin *et al.* 1998; Schumacher, Crum *et al.* 2004; Schumacher, Rivard *et al.* 2001; Yellen 2002) suggest that on Ca²⁺ binding to the N-lobes of CaM, a dimeric complex may be formed between two adjacent CaM binding domains and Ca²⁺-CaMs, inducing a rotation that is transmitted to the corresponding S6 helices and opens the K_{Ca}3.1 channel gate. However, by far, little is known about the precise mechanism by which Ca²⁺ binding to the CaM-prebound K_{Ca}3.1 channel results in the opening of this channel.

1.5 K_{Ca}3.1 channel assembly, trafficking, targeting

As mentioned above, K_{Ca}3.1 channel is a tetrameric membrane protein and the normal channel function is available only if the complete channel is expressed at the cell surface. Previous studies showed that K⁺ channel assembly is regulated by specific domains either in the N or C-terminus (Shen, Chen *et al.*

1993). Recently, Jones and colleagues investigated the functional role of the N-terminal leucine zippers as well as a dileucine motif (Jones, Hamilton *et al.* 2004). The dileucine motifs are known to be involved in protein recycling and leucine zippers are known to be involved in protein-protein interactions (Francis, Jones *et al.* 1999; Garippa, Johnson *et al.* 1996; Kobe and Deisenhofer 1994; Melvin, Marsh *et al.* 1999; Petris, Camakaris *et al.* 1998). They showed that deletion of the cytoplasmic N-terminus of hK_{Ca}3.1 results in an inability of the truncated channel to traffic properly to the cell surface. Moreover, both the N-terminal dileucine motif and one of the leucine zippers are critical for maintaining K_{Ca}3.1 protein expression and function. The leucine zipper is also required for efficient channel tetramerization. Moreover, the C-terminal is also essential for the function of hK_{Ca}3.1 channel (Syme, Hamilton *et al.* 2002). In addition, Joiner and colleagues (Joiner, Khanna *et al.* 2001) demonstrated that the assembly of calmodulin with the proximal C-terminal CaM binding domain is required for the targeting of hK_{Ca}3.1 to the plasma membrane. This was due to an enhanced assembly of channel protein into tetramers in the presence of calmodulin.

1.6 K_{Ca}3.1 channel pharmacology

K_{Ca}3.1 channel can be directly blocked by the antifungal drug clotrimazole (IC₅₀: 60 nmol/L), which also blocks CYP₄₅₀. Therefore, clotrimazole is not a selective K_{Ca}3.1 blocker (Alvarez, Montero *et al.* 1992; Brugnara, Gee *et al.* 1996; Fanger, Ghanshani *et al.* 1999; Jensen, Odum *et al.* 1999; Khanna, Chang *et al.* 1999; Vandorpe, Shmukler *et al.* 1998) (Figure 1.4 A). By substituting the imidazole ring by a pyrazole ring, Wulff and colleagues designed a much more specific and even more potent K_{Ca}3.1 channel blocker named TRAM-34 (IC₅₀:

20 nmol/L) (1-[(2-chlorophenyl) diphenylmethyl]-1*H*. pyrazole) (Figure 1.4 B) (Wulff, Miller *et al.* 2000), which is now widely used as a powerful pharmacological tool for this channel. Further studies showed that the two internal threonine and valine residues just below the selectivity filter are responsible for the $K_{Ca3.1}$ channel sensitivity to clotrimazole and TRAM-34 (Wulff, Gutman *et al.* 2001). In addition, the $K_{Ca3.1}$ channel is blocked by the scorpion peptide toxin charybdotoxin (IC_{50} : 5 nmol/L) which is also a blocker for $K_{Ca1.1}$ (Ishii, Silvia *et al.* 1997; Joiner, Wang *et al.* 1997; Logsdon, Kang *et al.* 1997). The $K_{Ca3.1}$ channel can be activated by 1-ethyl-2-benzimidazolinone (1-EBIO) and its much more potent derivative 5, 6-dichloro-1-ethyl-2-benzimidazolinone (DC-EBIO), both of which enhance the channel activity by keeping the channel in the open state (Figure 1.4 C).

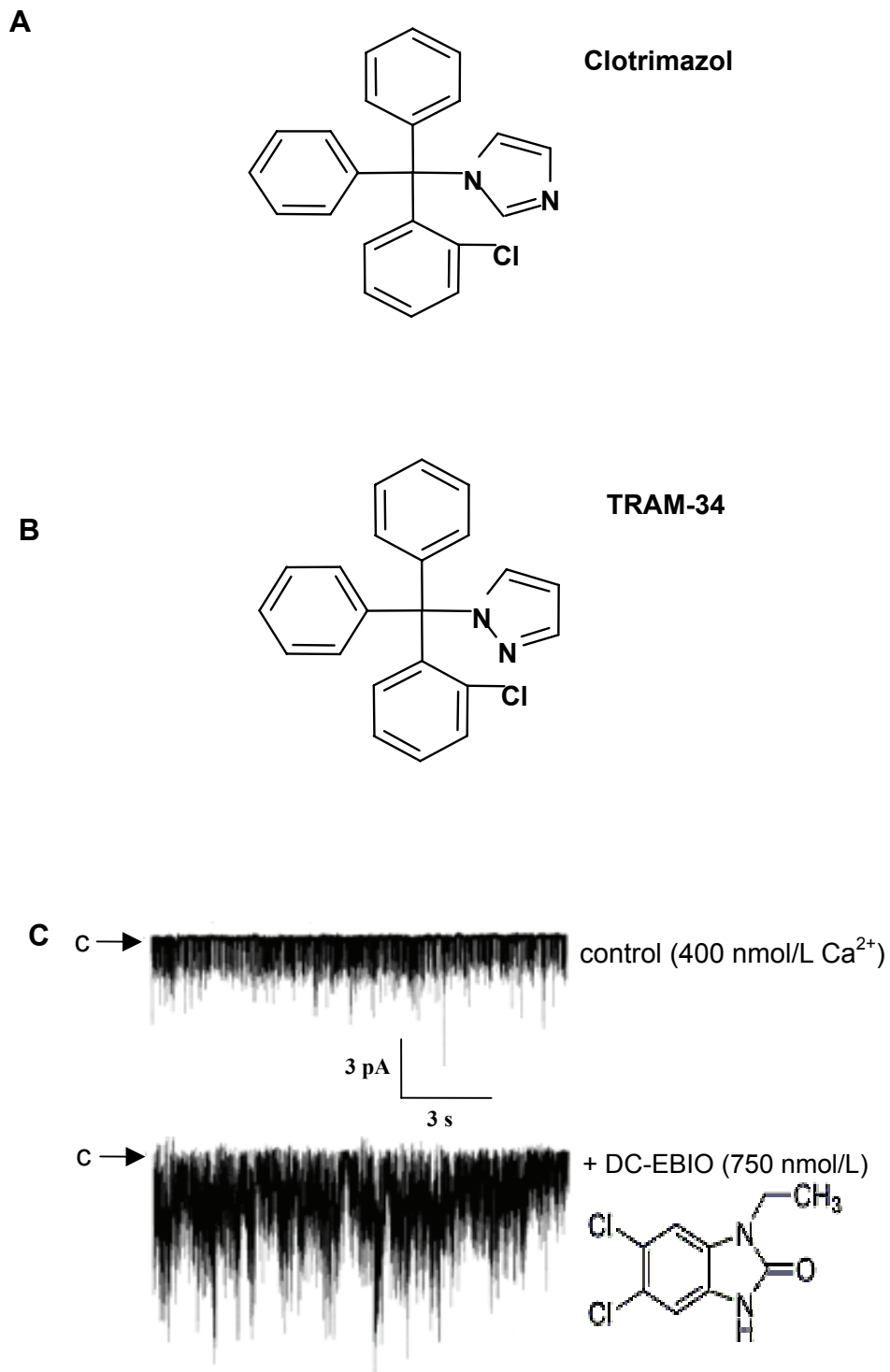


Figure 1.4 Pharmacological profile of $K_{Ca3.1}$ channel. **A.** $K_{Ca3.1}$ blocker clotrimazole. **B.** Newly synthesized $K_{Ca3.1}$ selective blocker TRAM-34. **C.** Single $hK_{Ca3.1}$ channel traces recorded from an inside-out cell patch at -80 mV. Single channel current was dramatically increased by addition of 750 nmol/L channel activator DC-EBIO. c indicates the close state of the channel.

1.7 $K_{Ca}3.1$ channel in the vasculature

The $K_{Ca}3.1$ channel is expressed in many organs and tissues such as placenta (Mohammed, Stulc *et al.* 1993), prostate (Ishii, Silvia *et al.* 1997), liver (Joiner, Wang *et al.* 1997), intestine (Ishii, Silvia *et al.* 1997; Logsdon, Kang *et al.* 1997), erythrocytes (Gardos 1958) and T-lymphocytes (Ghanshani, Wulff *et al.* 2000; Wulff, Calabresi *et al.* 2003), fibroblasts (Pena and Rane 1999), colonic epithelium (Devor, Singh *et al.* 1996). Importantly, $K_{Ca}3.1$ channel is expressed in endothelium of blood vessels from human and other species (Ghanshani, Coleman *et al.* 1998; Ishii, Silvia *et al.* 1997; Joiner, Wang *et al.* 1997; Kohler, Brakemeier *et al.* 2001; Kohler, Degenhardt *et al.* 2000; Logsdon, Kang *et al.* 1997; Meyer, Schonherr *et al.* 1999). Moreover, this channel is not distributed in differentiated mature vascular smooth muscle cells (VSMCs), but in proliferating neointimal VSMCs (Kohler, Wulff *et al.* 2003).

1.7.1 K_{Ca} channels and endothelial function

The endothelium is an important regulator of vascular tone. Circulating humoral factors, such as acetylcholine and bradykinin as well as hemodynamic forces (shear stress) elicited by the the streaming blood evoke the synthesis and release of vasodilators. Prostacyclin (PGI_2) and nitric oxide (NO) have been first identified as important endothelial-derived vasodilators (Furchgott and Vanhoutte 1989; Furchgott and Zawadzki 1980; Palmer, Ferrige *et al.* 1987).

In the late 1980s, a third vasodilating system was postulated which is distinct from NO and PGI_2 , since blockers of NO and PGI_2 synthesis do not fully suppress endothelium-dependent vasodilation. This type of vasodilation was firstly investigated in porcine and canine arteries exposed to acetylcholine or bradykinin (Beny and Brunet 1988; Bolton, Lang *et al.* 1984; Chen, Suzuki *et al.*

1988; Feletou and Vanhoutte 1988). The factor involved in this endothelium-dependent vasodilation was termed endothelium-derived hyperpolarizing factor (EDHF) since it causes hyperpolarization of the vascular smooth muscle (Brandes, Schmitz-Winnenthal *et al.* 2000; Feletou and Vanhoutte 2000; Nakashima, Mombouli *et al.* 1993), thus leading to a decrease in smooth muscle intracellular Ca^{2+} and consequently to vasodilation (Nelson, Patlak *et al.* 1990). While the contribution of EDHF to endothelium-mediated vasodilation is relatively small in large arteries, the EDHF appears to become more important as vessel diameter decreases. Especially, in small (resistance) arteries and in the microcirculation, EDHF is considered as important as or even more important than the two “classical” endothelium-derived relaxing factor, NO and PGI_2 (Brandes, Schmitz-Winnenthal *et al.* 2000; Tomioka, Hattori *et al.* 1999).

The exact nature of EDHF and EDHF-signaling was unclear yet for considerable time. Some investigators hypothesized that EDHF is a chemical factor released by the endothelium. Metabolites of arachidonic acid, i.e. cytochrome P450 epoxygenase-generated EETs have been postulated to mediate EDHF-signaling by activating large-conductance K_{Ca} in VSMCs of i.e. pig coronary arteries (Fleming, Fisslthaler *et al.* 1996; Lischke, Busse *et al.* 1995). In other vessels and species EETs do not seem to be involved in EDHF signaling (Eichler, Wibawa *et al.* 2003), thus questioning whether or not EETs are the sole EDHF. Other investigators hypothesized that EDHF-signaling is more likely a physical event characterized by the electrical spread of hyperpolarization from the endothelium to the vascular smooth muscle (Griffith 2004).

Recent studies revealed that opening of endothelial K_{Ca} channels is a hallmark in the generation of the EDHF-type vasodilation (Eichler, Wibawa *et al.* 2003; Griffith 2004; Kohler, Eichler *et al.* 2005; Marrelli, Eckmann *et al.* 2003;

Petersson, Zygmunt *et al.* 1997). Activation of these K_{Ca} channels is evoked by an elevation of $[Ca^{2+}]_i$ which is due to the Ca^{2+} release from the endoplasmic reticulum (ER) and subsequent Ca^{2+} entry from extracellular space (Marchenko and Sage 1993; Nilius, Droogmans *et al.* 2003; Sedova, Klishin *et al.* 2000). The opening of K_{Ca} channels leads to hyperpolarization of endothelial cells (see scheme in Figure 1.5) (Edwards, Dora *et al.* 1998; Frieden, Sollini *et al.* 1999; Gordon and Martin 1983; Mehrke and Daut 1990). Thereafter, the endothelial hyperpolarization is transmitted to vascular smooth muscle cells (VSMC) either by K_{Ca} -mediated K^+ release and subsequent activation of inwardly rectifying K^+ channels and Na^+/K^+ -ATPases (Blanco and Mercer 1998; Zaritsky, Eckman *et al.* 2000) or via myoendothelial gap junctions (Figure 1.5) (Ghanshani, Coleman *et al.* 1998). However, the precise mechanism of EDHF-signaling is still under debate (Griffith 2004).

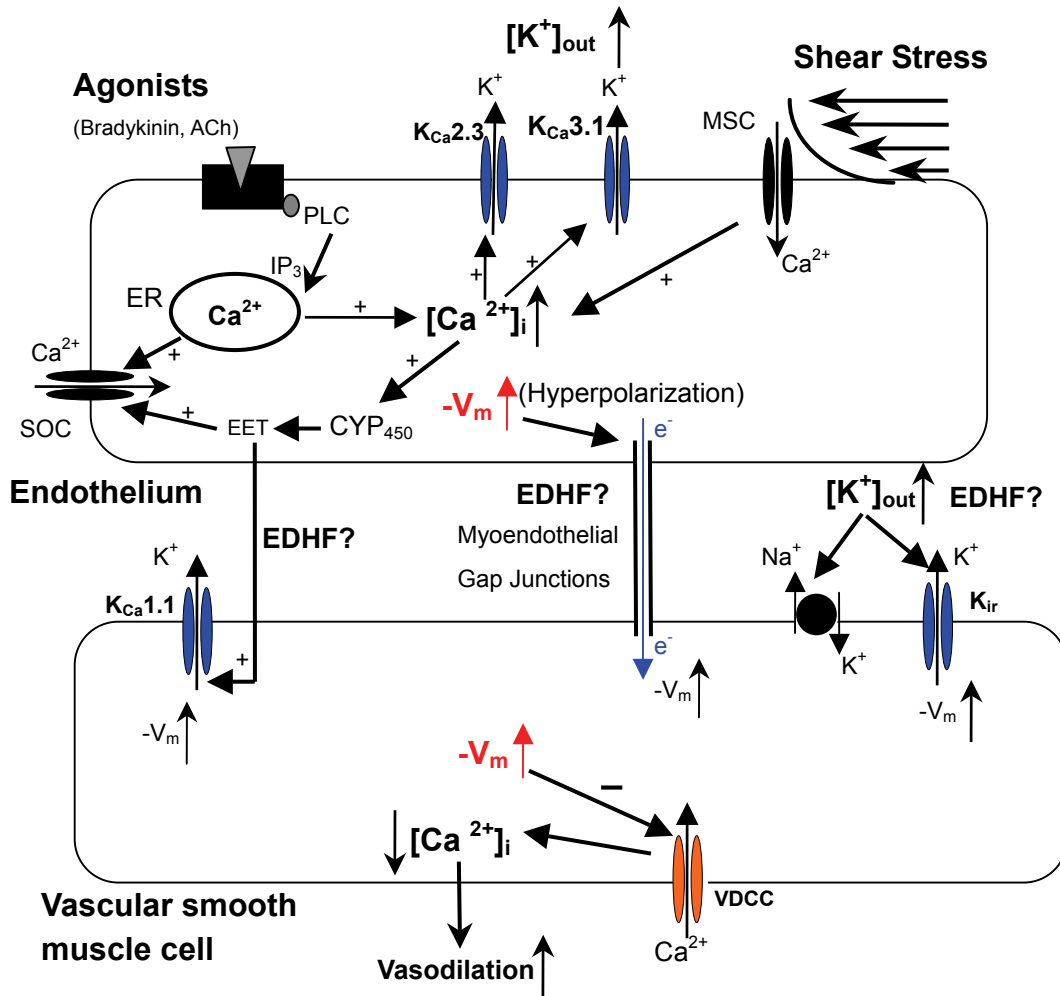


Figure 1.5 Endothelium-dependent vasodilatation and K^+ channels. The Ca^{2+} release from internal store, which stimulated by humoral factors such as acetylcholine and bradykinin, increases the $[Ca^{2+}]_i$ level which in turn activates endothelium $K_{Ca}3.1$ and $K_{Ca}2.3$ channel to produce the hyperpolarization of the EC cell membrane. Shear stress-induced mechanosensitive Ca^{2+} entry may also contribute to the elevation of the $[Ca^{2+}]_i$. The hyperpolarization is communicated from endothelium to VSMC probably via (1) EET, (2) endothelium-derived K^+ , (3) myoendothelial gap junctions. VDCC is inhibited by the endothelium-derived hyperpolarization, resulting in a decrease of the $[Ca^{2+}]_i$ in the VSMC and thus a vasodilation effect. PLC indicates phospholipase C; IP₃, 1, 4, 5-inositol-trisphosphate; ER, endoplasmic reticulum; SOC, store operated-channel; CYP₄₅₀, cytochrome *P*₄₅₀ epoxygenase; EET, epoxyeicosatrienoic acid; MSC, mechanosensitive cation channel; $-V_m$, negative membrane potential; VDCC, voltage-dependent Ca^{2+} channel; K_{Ca} , Ca^{2+} activated K^+ channel; K_{ir} , inwardly rectifying K^+ channel. + means activation; -, deactivation.

1.7.2 $K_{Ca}3.1$ channels and EDHF-signaling

Previous experiments of our own group (Eichler, Wibawa *et al.* 2003) revealed that EDHF-mediated vasodilation can be almost completely abolished by the administration of highly selective blockers of $K_{Ca}3.1$ and $K_{Ca}2.3$ (TRAM-34 and apamin) in rat carotid artery (CA). The sole blockage of one K_{Ca} channel was not sufficient to suppress EDHF-signaling. Thus this indicates that $K_{Ca}3.1$ - and $K_{Ca}2.3$ -produced hyperpolarization is essential for the EDHF-type vasodilation in rat CA. Moreover, our studies showed that arachidonic acid metabolites (EETs) do not contribute to the EDHF-mediated vasodilations in rat CA. Meanwhile, similar results were obtained by other groups investigating other vessel types and species (Petersson, Zygmunt *et al.* 1998; Vanheel and Van de Voorde 1997; Zygmunt, Edwards *et al.* 1996).

Thus until now there is convincing evidence that both $K_{Ca}3.1$ and $K_{Ca}2.3$ mediate endothelial hyperpolarization and thereby initiate EDHF-signaling. However to which extent $K_{Ca}3.1$ alone or together with $K_{Ca}2.3$ is in fact involved in EDHF-signaling is not clear. Moreover, the contribution of EDHF-type vasodilation to the control of vascular tone and systemic blood pressure is not clear.

1.8 Gene targeting in the murine genome

The term “gene targeting” or “knockout” denotes a technology to introduce precise genetic alterations into the genome of the mouse and to produce an animal model with a predefined genetic defect.

Eukaryotic cells have the intrinsic ability to exchange sequences between two homologous duplex molecules of DNA. This so-called “homologous recombination” event results in a reciprocal exchange of genetic information, taking place not only between homologous chromosomes but also between chromosomes and extrachromosomal lineated plasmid molecules (e.g. targeting constructs) that have been transfected into cells (Capecchi 1989). Because homologous recombination is a rare event, the positive/negative selection scheme developed by Capecchi and coworkers has been widely employed for gene targeting experiments. In brief, a targeting construct is assembled that contains part of the target gene separated by a positive selection marker, which is resistant to neomycin. A negative selection marker is placed downstream of those target gene sequence in the construct. So once the construct is homologously integrated into the genome, the positive selection marker will remain in the gene whereas the negative selection marker is gone. Using this positive/negative selection strategy, targeting constructs can be introduced into any gene for which homologous sequences are available.

Since about two decades ago, the field of mouse genetics has been revolutionised by the establishment of embryonic stem (ES) cell technology. ES cells are derived from the early mouse embryo and can be maintained for a prolonged period in culture in an undifferentiated state (Bradley et al., 1984). However, upon return to the environment of the early embryo, they can contribute to all cell types of the resulting chimera, including germ cells. Thus,

any genetic alteration that can be introduced into cultured ES cells can in principle be transferred to the germline to study of its effects in the intact animal after appropriate breeding. In detail, ES cell clones heterozygous for a gene disruption are isolated by positive/negative selection as mentioned above, and are subsequently injected into the blastocoel cavity of mouse embryos at day 3.5 of gestation using micromanipulation. These blastocysts are in turn transferred into pseudo-pregnant foster mothers to develop to term. The chimeric animals obtained are bred to normal mice and their offspring are tested for the presence of the mutant allele that has been transmitted through the germ line. F1 generation animals, which are heterozygous for the gene alteration, can then be bred to each other to derive mice homozygous for the mutant allele.

The first targeted mutation in ES cells was reported in 1988 (Doetschman, Maeda *et al.* 1988; Mansour, Thomas *et al.* 1988) and the first mouse with a targeted mutation was created in 1989 (Thompson, Clarke *et al.* 1989). Since then, hundreds of such experiments have been successfully performed using different ES cells (Simpson, Linder *et al.* 1997) and mouse databases are available on the Internet (e.g. <http://www.jax.org>) (Sikorski and Peters 1997). However, conventional gene disruption still has some disadvantages. For instance, embryonic lethality caused by disruption of developmentally essential genes precludes the analysis of gene function in adult animals. Hence, it might be desirable to induce a gene defect only at distinct stage or in a tissue-specific manner. Conditional gene inactivation is based on the activity of site-specific recombinases (e.g. *Cre*) which specifically bind to a short recognition sequence (e.g. *loxP*, 34 bp) (Segev and Cohen 1981; Sternberg 1981). If two *loxP* sites are located in the same orientation on a DNA molecule, *Cre* recombinase-mediated recombination will result in excision of the intervening DNA region, leaving a single *loxP* site behind. Thus, a typical construct for

conditional gene targeting experiment contains a region of the target gene modified by integration of the positive selection marker and one loxp site in one intron and another loxp site in an adjacent intron. The modified introns flank an essential exon of the target gene. Using standard protocols, this modification is introduced into ES cells via homologous recombination and mice carrying two copies of loxp modified gene are produced by breeding. These animals are phenotypically normal. Tissue-specific gene inactivation can be achieved by crossing *loxp*-modified mice with transgenic mice carrying the *Cre* recombinase gene under the control of tissue-specific promoter elements (Gu, Marth *et al.* 1994).

1.9 Aims

The present study was designed to clarify the functional role of $K_{Ca3.1}$ channel in the vasculature, especially, in the endothelium. Previous studies demonstrated that $K_{Ca3.1}$ channel, together with other Ca^{2+} -activated K^+ channels, contribute to the endothelium-derived hyperpolarizing factor (EDHF)-mediated vasodilation of isolated vessels by inducing hyperpolarization of the endothelial membrane potential (Eichler, Wibawa *et al.* 2003; Kohler, Brakemeier *et al.* 2001; Kohler, Eichler *et al.* 2005). Thus this channel appears to be important in endothelial function and thereby in endothelial control of vascular tone. However, the precise role of $K_{Ca3.1}$ channel in the mechanisms of endothelium-dependent vasodilation and thus blood pressure control is still unclear.

Specific aims of this study:

- 1 Generation of the $K_{Ca3.1}$ knockout ($K_{Ca3.1}^{-/-}$) animal model, which allows determining the precise role of $K_{Ca3.1}$ in endothelial function,

EDHF-signaling and the control of vascular tone *in vivo*. $K_{Ca}3.1^{-/-}$ mice will be generated by using conventional and conditional gene targeting technologies. Successful gene targeting will be examined by PCR and southern blot analysis. Loss of functional $K_{Ca}3.1$ channel expression in knockout mice is confirmed by the use of patch-clamp technique.

- 2 General characterization (phenotyping) of $K_{Ca}3.1^{-/-}$ animal. This characterization includes the investigation of viability and fertility of $K_{Ca}3.1^{-/-}$ mice and gross morphological and histological examination of major organs. In case of early embryonic lethality of $K_{Ca}3.1^{-/-}$ mice generated by the conventional gene targeting strategy, the conditional strategy will be used to generate an inducible $K_{Ca}3.1^{-/-}$ animal model.
- 3 Cardiovascular phenotyping. The major aim of the present study is to determine the role of $K_{Ca}3.1$ in vasculature and especially in the endothelium. For this purpose, the following investigations are planned:
 - a) Characterization of the K_{Ca} currents in endothelium in knockout mice. By use of the patch-clamp technique, the lack of endothelial $K_{Ca}3.1$ current will be demonstrated. Moreover, it will be investigated whether the lack of $K_{Ca}3.1$ will lead to reduced K_{Ca} -currents and whether the lack of $K_{Ca}3.1$ might be compensated by other endothelial K_{Ca} channels such as $K_{Ca}2.3$. To test this, $K_{Ca}3.1$ and $K_{Ca}2.3$ current densities and their respective contribution to total K_{Ca} currents will be determined in freshly isolated aortic endothelial cells (ECs) from wild-type mice and $K_{Ca}3.1^{-/-}$ mice.
 - b) Functional consequences of the loss of $K_{Ca}3.1$ in ECs from $K_{Ca}3.1^{-/-}$ mice. Hence, it will be tested whether the lack of $K_{Ca}3.1$ in endothelium leads to a reduced K_{Ca} channel-mediated vasodilation (EDHF-signaling) and thus endothelial dysfunction. EDHF-mediated vasodilation is characterized by pressure myography in CA. Furthermore, to elucidate a

possible impaired EDHF-type vasodilation caused by $K_{Ca}3.1$ gene disruption and the consequence of such a possible endothelial dysfunction, blood pressure measurements e.g. non-invasive tail-cuff as well as telemetry will be performed in wild-type and $K_{Ca}3.1^{-/-}$ mice.