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its mutations, disease and treatment “

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Abstract

Inwardly rectifying potassium channels can be found in almost every part of the human body. Depending on their location in the cell, Kir channel family members carry out central tasks in numerous physiological processes, such as blood vessel constriction and shaping of the cardiac action potential.

The focus of this thesis lies on Kir1.1, more commonly referred to as ROMK: the renal outer medullary potassium channel. ROMK's essential role in processes like potassium recycling in the TAL or in ion transport in CCD cells make the channel a key player in the regulation of potassium and salt homeostasis in the kidney.

The discovery of the genetic basis of Bartter syndrome type II, a rare autosomal recessive salt-wasting nephropathy caused by loss-of-function mutations in ROMK, undermined the channel's clinical significance. The hereditary tubulopathy is associated with various metabolic abnormalities, for example hypokalemic metabolic alkalosis and hypercalciuria. Even though people suffering from the disease usually present during the neonatal period, in recent years several late-onset cases have been described.

By comparison of 16 case reports from 22 Bartter patients around the world, the following conclusions can be drawn: As a result of the disease being caused by numerous different mutations in the KCNJ1 gene, Bartter syndrome type II presents in many ways, which makes it even harder to diagnose. Often patients receive the correct diagnosis after suffering from the disease for multiple years, usually receiving misdiagnosis beforehand, which leads to believe that the number of Bartter patients is higher than expected.

Zusammenfassung

Einwärts gleichrichtende Kaliumkanäle sind in fast allen Bereichen des menschlichen Körpers zu finden. Je nach Lage in der Zelle übernehmen Mitglieder der Kir-Kanal-Familie zentrale Aufgaben bei zahlreichen physiologischen Prozessen, wie beispielsweise der Verengung von Blutgefäßen und der Gestaltung des kardialen Aktionspotentials.

Der Fokus dieser Arbeit liegt auf dem Kir1.1 Kanal, besser bekannt als ROMK: der renale äußere medulläre Kaliumkanal. Die essenzielle Rolle, die ROMK bei Prozessen wie dem Kalium-Recycling im TAL oder dem Ionentransport in CCD-Zellen spielt, machen den Kanal zu einem Hauptakteur bei der Regulierung der Kalium- und Salzho-möostase in der Niere.

Die Entdeckung der genetischen Grundlage des Bartter-Syndroms Typ II, einem seltenen autosomal-rezessiv vererbten Salzverlust-Syndrom, das durch Funktionsverlustmutationen in ROMK verursacht wird, unterstreicht die klinische Bedeutung des Kanals wesentlich. Die Tubulopathie geht mit verschiedenen Stoffwechselanomalien einher, wie beispielsweise der hypokaliämischen metabolischen Alkalose und Hyperkalziurie. Obwohl die Krankheit in der Regel bei Neugeborenen auftritt, wurden in den letzten Jahren mehrere Fälle mit späterem Auftreten, sogar im Erwachsenenalter, beschrieben.

Aus dem Vergleich von 16 Fallberichten verschiedener Bartter-Syndrom Typ II Patienten aus aller Welt lassen sich folgende Schlussfolgerungen ziehen: Da die Krankheit durch zahlreiche verschiedene Mutationen im KCNJ1-Gen verursacht wird, zeigt sich das Bartter-Syndrom Typ II auf vielfältige Weise, was die Diagnose noch schwieriger macht. Oft erhalten die Patienten die richtige Diagnose erst, nachdem sie mehrere Jahre an der Krankheit gelitten haben, wobei sie in der Regel zuvor eine Fehldiagnose erhielten. Dies lässt annehmen, dass die Anzahl an Bartter-Patienten höher ist als vermutet.

Table of Content

1	<u>OVERVIEW OF INWARDLY RECTIFYING K⁺ CHANNELS</u>	- 1 -
1.1	INTRODUCTION	- 1 -
1.2	THE 7 KIR FAMILIES, THEIR EXPRESSION & DISTRIBUTION	- 4 -
1.3	STRUCTURAL & FUNCTIONAL FEATURES	- 6 -
1.3.1	KIR CHANNEL GATING & ACTIVATION	- 7 -
1.3.2	KIR CHANNEL TRAFFICKING	- 8 -
1.3.3	SELECTIVITY FILTER	- 9 -
1.3.4	INWARD RECTIFICATION	- 9 -
1.4	KIR CHANNELOPATHIES	- 11 -
2	<u>THE RENAL OUTER MEDULLARY POTASSIUM CHANNEL</u>	- 13 -
2.1	INTRODUCTION TO ROMK	- 13 -
2.2	THE SIGNIFICANT ROLE OF ROMK IN THE KIDNEY	- 14 -
2.3	ROMK IN THE BRAIN	- 16 -
2.4	ROMK ISOFORMS	- 17 -
2.4.1	PHOSPHORYLATION BY PKC VARIES IN ROMK ISOFORMS	- 18 -
2.5	ROMK REGULATION & INTRACELLULAR LOCALIZATION	- 19 -
2.5.1	SURFACE EXPRESSION SUPPRESSORS	- 20 -
2.5.1.1	Ubiquitination	- 20 -
2.5.1.2	WNKs	- 20 -
2.5.2	SURFACE EXPRESSION ENHANCERS	- 22 -
2.5.2.1	SGK1	- 22 -
2.5.2.2	PKA	- 23 -
2.5.2.3	PKC	- 24 -
2.6	STRUCTURE-FUNCTION RELATIONSHIPS	- 25 -
2.6.1	POTASSIUM SELECTIVITY	- 26 -
2.6.2	POTASSIUM CONDUCTION	- 26 -
2.6.3	RAPID GATING	- 27 -
2.6.4	INWARD RECTIFICATION	- 28 -
2.6.5	REGULATION OF GATING	- 28 -
2.7	THE CYTOPLASMIC DOMAIN	- 30 -

2.7.1	PORE EXTENSION	- 30 -
2.7.2	THE G-LOOP	- 31 -
2.8	MODULATION OF THE ROMK CHANNEL.....	- 33 -
2.8.1	ATP	- 33 -
2.8.2	PIP2.....	- 33 -
2.8.3	PH SENSITIVITY	- 35 -
2.8.3.1	The RKR Triad.....	- 35 -
2.8.3.2	The hydrophobic residue L160.....	- 36 -
2.8.3.3	Residues in the TM2 transmembrane region.....	- 36 -
2.8.3.4	TM1–TM2 H bonding.....	- 37 -
2.9	PHARMACOLOGY	- 39 -
2.9.1	NATURAL TOXINS AS ROMK BLOCKERS.....	- 39 -
2.9.2	SMALL-MOLECULE ROMK INHIBITORS	- 39 -
3	<u>BARTTER SYNDROME TYPE II.....</u>	<u>- 45 -</u>
3.1	INTRODUCTION	- 45 -
3.2	TREATMENT	- 46 -
3.2.1	ORAL SALT SUPPLEMENTATION	- 46 -
3.2.2	NON-STEROIDAL ANTI-INFLAMMATORY DRUGS (NSAIDs)	- 46 -
3.2.3	POTASSIUM-SPARING DIURETICS.....	- 47 -
3.2.4	RENIN-ANGIOTENSIN-ALDOSTERONE SYSTEM INHIBITORS.....	- 47 -
3.3	ANALYSIS OF 22 BS2 PATIENTS	- 49 -
3.3.1	CASE REPORT REFERENCES	- 49 -
3.3.2	CASE REPORT TABLE	- 50 -
3.3.3	CASE REPORT ANALYSIS.....	- 51 -
3.3.4	ANALYSIS OF CASE REPORT MUTATIONS.....	- 52 -
3.4	STRUCTURAL INTERPRETATION	- 55 -
3.5	PREDICTION OF VARIANT PATHOGENICITY	- 56 -
4	<u>CONCLUSION</u>	<u>- 57 -</u>
5	<u>APPENDIX.....</u>	<u>- 58 -</u>
5.1	LIST OF FIGURES	- 58 -

1 OVERVIEW OF INWARDLY RECTIFYING K⁺ CHANNELS

1.1 INTRODUCTION

In 1949 the german physician and biophysicist Bernhard Katz observed in skeletal muscle fibers of frogs, that the membrane conductance of cells at hyperpolarizing potentials was larger, when these cells were dipped in solutions with high potassium concentration.^{1,2} This performance was contradictory to what Cole and Curtis had observed in squid giant axon, where they distinguished the depolarization-induced non-linear conductance as rectification.³ Hodgkin and Huxley, working with Katz in his laboratory, exhibited the first case of outward rectification, demonstrated by the delayed rectifier K_v current.^{2,4} As a consequence, Katz named the skeletal muscle conductance in high K⁺ “anomalous rectification”.² Later the name was changed to “inward rectification”, which is still to this day the term most commonly used for Kir channels.⁵ Outwardly rectifying K_v currents and Kir channels also differ in their voltage dependance, as inwardly rectifying potassium channels do not follow Hodgkin-Huxley kinetics.⁶ Kir channels are voltage independent. The driving force of Kir channel activity is the electrochemical potassium gradient, meaning the distance of V_m, the membrane potential, from E_K, the equilibrium potential, for potassium ions, in short: V_m - E_K.² If a shift in extracellular potassium concentration [K⁺]_o, but not in intracellular potassium concentration [K⁺]_i occurs, Kir channel stimulation is dependent on V_m - E_K.⁷ Intracellular divalent cations as well as other molecules block the channel pore asymmetrically, thereby causing these defining Kir channel characteristics.⁸

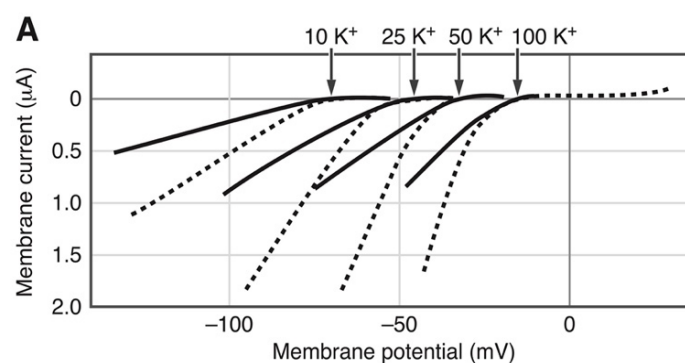


FIGURE 1: KIR CHANNEL CONDUCTANCE & RECTIFICATION⁸

Kir channel conductance and rectification depends on extracellular potassium concentration. I-V relationships of starfish egg cell membrane at four different [K⁺]_o: 10, 25, 50 and 100 mM in Na⁺ free media. Instantaneous and steady-state currents are displayed by continuous and dashed lines.^{8,9}

Consequently, they do not allow much current flow at potentials positive to E_K however, they achieve great potassium conductance at potentials negative to E_K , under physiological conditions.^{8,10,11} No spontaneous electrical activity and a resting membrane potential (E_{res}) in proximity to E_K are displayed by cells with great Kir conductance. This indicated feature along with the voltage independence of inwardly rectifying potassium channels, is why they play critical roles in managing the action potential duration in electrically excitable cells, for example cardiac muscle.^{8,10}

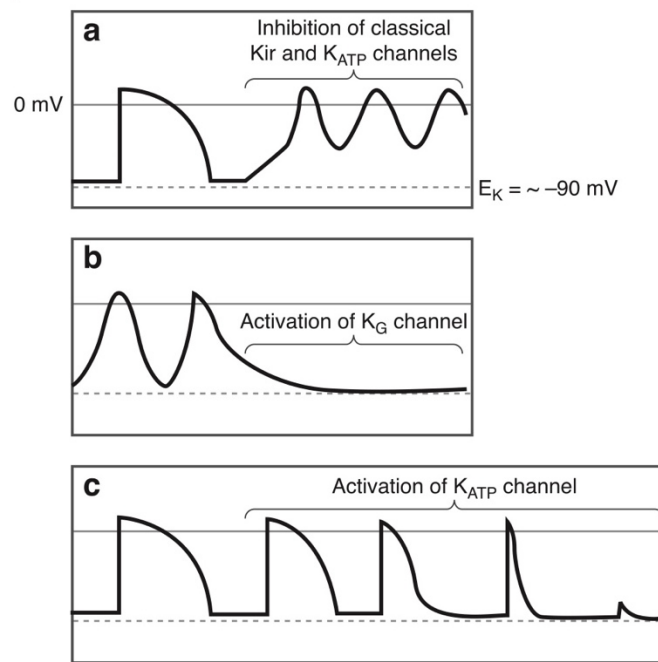


FIGURE 2: CONTROL OF EXCITABILITY IN CARDIAC CELLS BY KIR CHANNELS⁸

Action potentials under various conditions are displayed. a and c: in ventricular myocytes. b: in the sinoatrial node. a: On the one hand, blockage of Kir and K_{ATP} channels leads to depolarization of the resting membrane potential E_{res} and to fluctuation of the membrane potential E_m in ventricular myocytes. c: On the other hand, E_m is hyperpolarized, action potential duration reduced and action potential generation suppressed through K_{ATP} channel activation. b: Numerous K_G channels are expressed in the sinoatrial node, stimulation of those channels possibly causes E_m hyperpolarization and/or bradycardia.⁸

Kir channels can be found in numerous organs and tissues. They are expressed in various cells, such as cardiac myocytes,^{12,13,14,15} neurons,^{16,17,18,19,20,21} blood cells,^{22,23} osteoclasts,²⁴ endothelial cells,²⁵ epithelial cells,^{26,27,28,29} oocytes^{30,9} and glial cells^{10,31,32}. Inward rectification is further displayed by ATP-sensitive potassium channels, in short K_{ATP} channels and G protein-gated potassium channels, abbreviated K_G channels.⁸ The latter are stimulated by pertussis toxin (PTX)-sensitive G proteins. Channel localization in the cell, ion flux as well as pore opening regulation control physiological activities and tasks of Kir channels.⁸

In the late 1960ies and 1970ies inward rectification was traced back to channel block by cytosol molecules.² Later it was established that magnesium concentrations in the millimolar range and sub-millimolar polyamine concentrations inside the cells act as intracellular blockers. As a result of rapid magnesium unblock, the Kir channel inward current exhibited a time-independent boost after hyperpolarization. This was followed by a time-dependent boost caused by slow polyamine unblock.² Numerous intracellular proteins, ions, nucleotides, polyamines along with lipids manage the ion flow and opening of the pore.⁸ The functional role a Kir channel carries in different cells and tissues depends on where in a cell the channel is located, especially in which cell region, for example pre-or postsynaptic sites in neurons or apical or basolateral membranes in epithelial cells.

All members of the inward-rectifying potassium channel family share various structural features. Despite their equivalence in multiple functional properties, the channels each obtain individual characteristics in order to fulfill their central roles in numerous physiological processes including secretion of hormones, constriction of blood vessels, regulation of membrane potential and epithelial salt transport.³³ Moreover, some are critically involved in neuronal activity as well as the regulation of heart functions such as the shape of the cardiac action potential or the setting of the resting potential.³⁴ Since Kir channels are ubiquitously expressed, channel mutations resulting in functional defects are associated with several human diseases and syndromes. Kir channelopathies, which will be further discussed down below, are known to affect multiple organ systems including skeletal muscle, heart, kidney, the central as well as the peripheral neural system and the pancreas.³⁵

1.2 THE 7 KIR FAMILIES, THEIR EXPRESSION & DISTRIBUTION

The 16 human Kir channel isoforms were, on the basis of phylogenetic analysis and sequence alignment divided into seven subfamilies that can be classified into four functional groups:² Kir1.x to Kir7.x. Classical Kir channels (Kir2.x) are constitutively active, G protein-gated Kir channels (Kir3.x) are regulated by G protein-coupled receptors, ATP-sensitive K⁺ channels (Kir6.x) are tightly linked to cellular metabolism, and K⁺ transport channels (Kir1.x, Kir4.x, Kir5.x, and Kir7.x).⁸

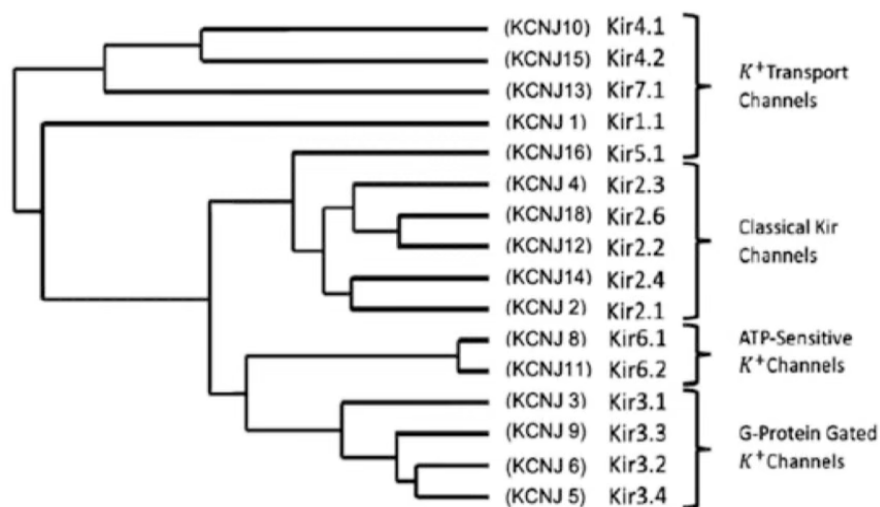


FIGURE 3: THE KIR CHANNEL PHYLOGENETIC TREE²

Based on amino acid sequence alignment and phylogenetic analysis, the 16 subunits of human Kir channels are classified into four functional groups: a. the K⁺ transport, b. the classical, c. the ATP-sensitive, and d. the G protein-gated K⁺ channels.²

Sequence similarity among Kir family members varies.² Homology in sequence is 30% between some subfamilies and rises to 99% within others, making the building of homomeric as well as heteromeric formations possible. Kir5.1 is able to associate across subfamilies with either Kir4.1 or Kir4.2 to form functional channels.³⁶ Usually though, assemblies within subfamilies are built, for example Kir3.1/3.2 can be found in brain tissue and Kir3.1/Kir3.4 in atrial cardiomyocytes.² Resulting heteromers and homomers differ in their functional properties. Although, compared to other K⁺ channels, Kir channels generally associate without accessory subunits, Kir6.x is an exception, as these channels build octamers, consisting of four pore-forming subunits as well as four auxiliary ATP/ADP sensing subunits of the sulfonylurea receptor, SUR1 or SUR2A.^{36,37}

Kir channel family members can be found in almost every part of the human body, hence their widespread expression pattern.^{35,38} Both, strong and weak rectifiers belong

to the family of inwardly rectifying potassium channels. In excitable cells, for example muscle or neuronal cells, usually strong rectifiers, like Kir2 and Kir3 can be found. These channels play a significant role in stabilizing the resting membrane potential as well as in repolarization. The rectifying features of these strong rectifying channels make it possible for cells to maintain potassium while the action potential is built. Additionally, potassium entry is promoted, following hyperpolarization of the cell.³⁵

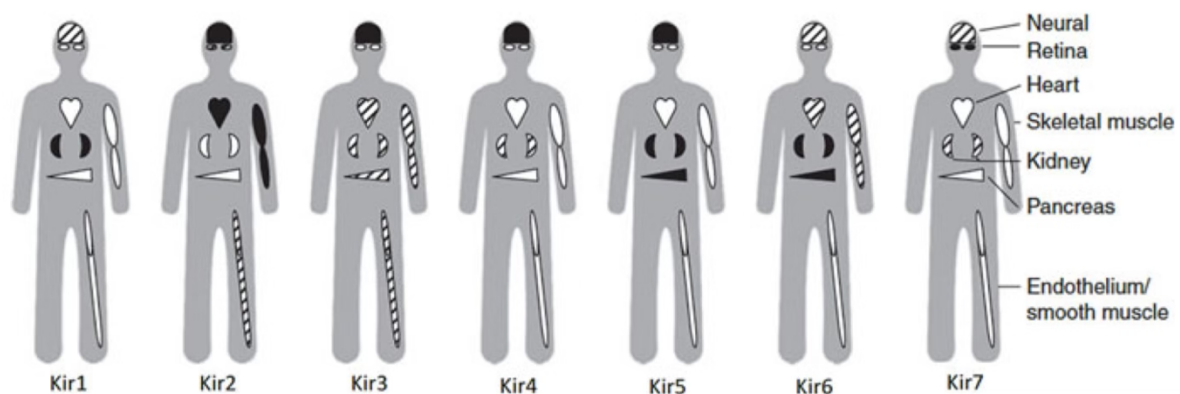


FIGURE 4: TISSUE DISTRIBUTION OF KIR CHANNELS²

Overview of organs and tissues where each of the seven Kir channel subfamily members is expressed. Blank means no expression, the striped boxes indicate intermediate expression and black displays high levels of expression.

Kir2, Kir3, Kir4 and Kir5 exhibit high expression in the brain, whereas Kir4, Kir5 and Kir6 are strongly expressed in the kidneys.³⁵ In the pancreas, expression of Kir5 and Kir6 is elevated. Kir2, Kir3 and Kir6 are strongly expressed in cardiomyocytes while Kir7 is highly expressed in the retina. In the heart, Kir2 displays reduced expression in the atrioventricular node and stronger expression in the ventricles. In functioning atrial as well as ventricular cardiomyocytes members of the Kir2 subfamily build the classical I_{K1} current. They play essential roles in stabilizing the resting membrane potential and repolarization in these heart cells. Kir3 (I_{KAch}) can be found in various brain areas, contributing to multiple neurological functions. In the heart, Kir3 can primarily be found in the atrium rather than in the ventricle. Moreover, these channels are widely expressed in nodal heart tissues, participating in regulation of the heart rate.³⁵ Weak rectifiers, such as Kir1, Kir4 and Kir5 are essential for managing potassium homeostasis.

1.3 STRUCTURAL & FUNCTIONAL FEATURES

Via expression-cloning techniques the first isolation of two Kir channel cDNAs was accomplished in 1993.⁸ From the outer medulla of rat kidney and a mouse macrophage cell line ROMK1, the renal outer medullary potassium channel, also known as Kir1.1 which is an ATP-dependent Kir channel³⁹ and IRK1 or Kir2.1⁴⁰ which is known as the classical Kir channel, were isolated. Through comparison of the primary structures of these two channels many similarities were found.⁸ An extracellular pore-forming region, the ion selectivity filter (H5), builds the bridge between two putative membrane spanning domains, commonly referred to as TM1 and TM2. One is associated with an amino- and the other with a carboxy terminal domain. It was later discovered that every member of the inward rectifier potassium channel family is in possession of these features, then acknowledged as the basic building block of Kir channels.⁸

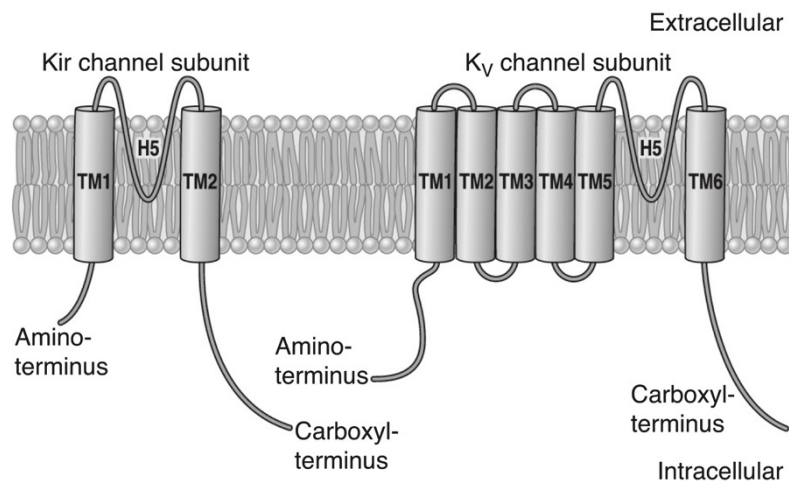


FIGURE 5: COMPARISON OF SUBUNITS: KIR CHANNEL VS. K_v CHANNEL⁸

The primary structure of the Kir channel subunit on the left and the primary structure of the voltage gated K^+ (K_v) channel subunit on the right. Every Kir subunit consists of two transmembrane regions (TM1 and TM2), a pore-forming loop (H5) and cytosolic amino and carboxy termini. For comparative purposes, the structure of the voltage gated K^+ (K_v) channel subunit is illustrated on the right, which consists of six transmembrane regions (TM1-TM6).⁸

In comparison to members of the voltage gated potassium channel family (K_v) the anatomy of Kir channels is rather simple,⁴¹ as K_v channels consist of six instead of two transmembrane domains. Their basic composition plays a key role in the pharmacology of the inward rectifier potassium channel family. As previously mentioned, Kir channels exist either as homotetramers or heterotetramers which consist of four of those just described subunits in a tetrameric complex, thereby forming the channel pore. The transmembrane domains of each subunit are divided by a pore-loop.³⁵ This pore-loop can be found near the extracellular membrane side, containing the GYG or

GFG potassium selectivity filter motif. The negatively charged residue E138 along with the positively charged residue R148 are responsible for poor-loop stability in the Kir2.1 channel. The characteristic cytoplasmic extended pore domain (CTD) is formed by a short amino-terminus connected to TM1 and a longer carboxy-terminus linked to TM2. Even though Kir channel family members resemble much in structure, they differ in various individual characteristics, such as response to regulatory signals and sensitivity to extracellular barium.³⁵

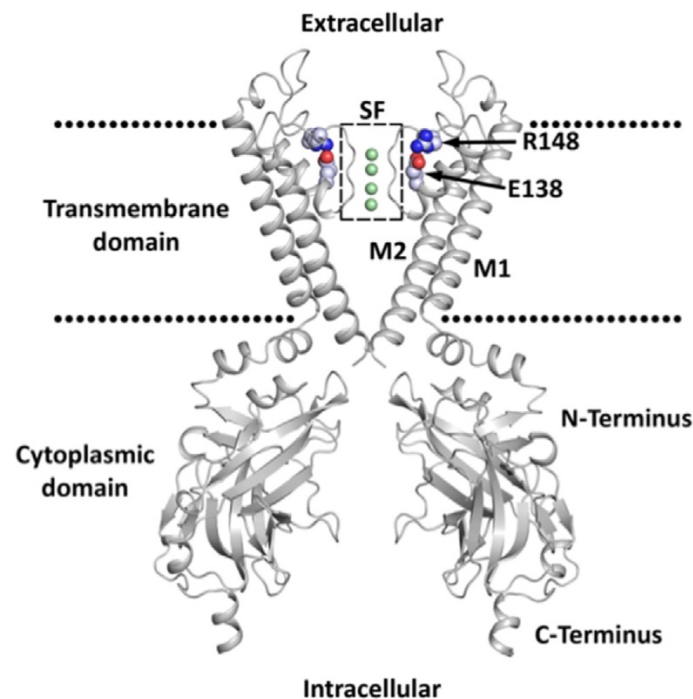


FIGURE 6: MODEL OF THE KIR2.1 CHANNEL³⁵

Displayed are two opposite Kir channel domains with common structural features highlighted. Dotted lines demonstrate the location of the membrane. A dotted box indicates the selectivity filter, with tiny green spheres illustrating ions inside the selectivity filter. Also shown as spheres are the residues responsible for poor-loop stability, E138 and R148.³⁵

1.3.1 KIR CHANNEL GATING & ACTIVATION

Two gating structures control intrinsic Kir channel gating: the G loop, located in the cytoplasmic domain and the TM2 bundle-crossing region of the transmembrane domain.³⁶ Apart from polyamines, magnesium and extracellular potassium concentrations, Kir channel gating is regulated by multiple other regulators, for example ATP, G proteins, pH and Na⁺. The signaling phospholipid phosphatidylinositol 4,5-bisphosphate, often referred to as PIP2, plays a significant role in Kir channel gating and activation. Based on their degree of stereospecificity and affinity for PIP2 over PI(3,4,5)P3 and PI(3,4)P2:⁴² *Highest or Group 1* (Kir2.1, Kir2.4, Kir4.1), *Moderate or Group 2*

(Kir1.1, Kir2.2, Kir2.3, Kir4.2, Kir7.1), *Weak* or *Group 3* (Kir3.4, Kir3.1/3.4), *Lowest* or *Group 4* (Kir6.2, Kir6.2/SURs). For Kir activation and stimulation by various endogenous Kir channel gating regulators, the existence of this membrane phospholipid in the inner surface of the plasma membrane is crucial.³⁶ These regulators vary among Kir channel family members. GIRK for instance, needs Na⁺ and Gβγ for activation, whereas ATP is crucial for activation of Kir6.x channels. In addition, arachidonic acid increases current flow via channels containing Kir2.3. A rise in intracellular concentration of long-chain CoA esters inhibits Kir2.1 but activates Kir6.x channels. Another membrane lipid that modifies activity of some Kir channels is cholesterol. It activates GIRK channels in dependence of PIP2 and independent of G-proteins, whereas rising cholesterol concentrations inhibit Kir2.x channels.³⁶

1.3.2 KIR CHANNEL TRAFFICKING

Kir channels are transmembrane proteins, therefore they are translated in the endoplasmic reticulum and afterwards carried to the plasma membrane through the Golgi apparatus and the trans-Golgi network.² This process is called forward trafficking. In the procedure known as backward trafficking, proteins enter the degradation pathway, after being removed from the plasma membrane. First Kir channels are trafficked to the early endosome and then to the multivesicular body. Final degradation steps take place in the lysosome. In addition, the channels can enter multiple recycling pathways instead. In this case, they are recycled back to the plasma membrane via the early endosome as well as the trans-Golgi network.² All of these recycling pathways are closely monitored, depending on individual trafficking motifs in the Kir channels primary sequence, interacting with specific proteins that are responsible for following trafficking actions.³⁵ Multiple channel mutations cause LOF: loss of function, or GOF: gain of function in the Kir trafficking process. These are associated with numerous severe human diseases, including Bartter syndrome type II, a LOF in the Kir1.1 channel or Keppen-Lubinsky syndrome, a GOF in the Kir3.2 channel.²

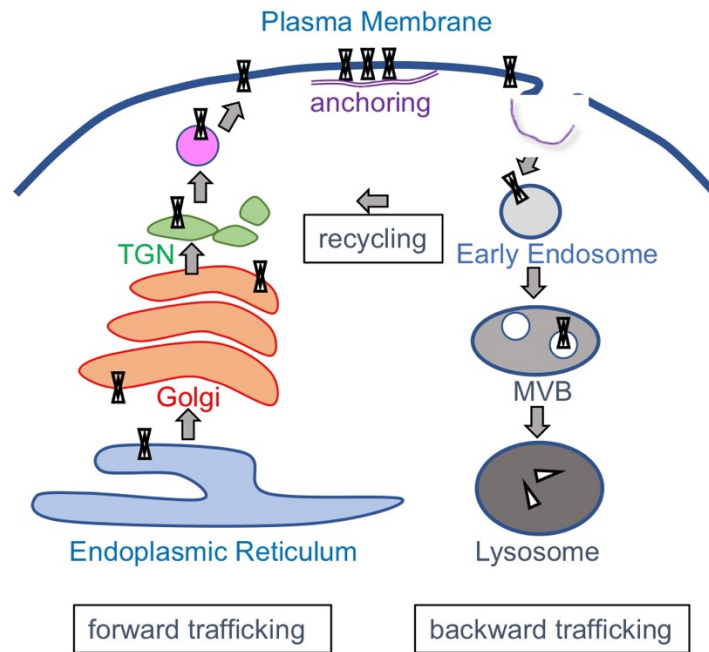


FIGURE 7: SCHEMATIC ILLUSTRATION OF KIR CHANNELS' INTRACELLULAR TRAFFICKING ROUTES³⁵

After Kir channels are translated in the endoplasmic reticulum, correctly folded ones are shipped to the plasma membrane. This procedure is known as forward trafficking. Backward trafficking entails the degradation of Kir channels, once removed from the plasma membrane. Abbreviations: TGN: trans-Golgi network, MVB: multivesicular body.³⁵

1.3.3 SELECTIVITY FILTER

The Kir channel selectivity filter (SF) is comparable with the one of other potassium channels, but the Kir channel sequence is TIGYGXR (X: Y/F/H, V/L, T, G), instead of the canonical filter sequence TXGYGDX (X: aliphatic amino acid).² Although in some exceptions the sequence differs. The conserved Kir channel, for example in Kir2.2 the residue R149 creates an ionized hydrogen bond with the pore helix residue E139. Moreover, surrounding the pore region (C123-C155), a highly conserved disulfide bond can be found. Kir channels contain a conserved 3-10 helical HGDL signature sequence, between the pore region and outer helix turntable.⁴³ However, again there are exceptions, as some channels alter in sequence, for example instead of HGDL, ROMK contains a HKDL signature sequence.²

1.3.4 INWARD RECTIFICATION

The most distinctive quality of Kir channels is their inward rectification.³⁶ They can be classified into strong, intermediate and weak rectifiers: All Kir2 and Kir3 channels for instance, belong to the strong rectifying group, whereas Kir4 channels are intermediate

rectifiers and Kir6 channels and ROMK/Kir1.1 are weak rectifying channels.² Voltage-gated Na^+ , Ca^{2+} and K^+ channels contain a conserved voltage-sensing S4 domain, that is missing in Kir channel structures, making them insensitive to membrane voltage.⁸ However, inward rectification displays voltage sensitivity. The distinctive Kir channel feature is regulated by naturally occurring polyamines, such as spermidine, spermine and putrescine, along with intracellular magnesium.^{36,44} The inner vestibule is blocked by polyamines and magnesium, at membrane potentials positive to the equilibrium potential of potassium ($E_K \approx -95 \text{ mV}$).³⁶ Thus, making just a small outward current possible. On the contrary, great inward potassium current is enabled at potentials negative to E_K , when magnesium and polyamines move out of the channel into the cell. The power of inward rectification in Kir channels is determined by the affinity of polyamines and magnesium for TM2 helix binding sites⁴⁵ as well as cytoplasmic domain binding sites. Furthermore, extracellular K^+ concentrations $[\text{K}^+]_o$ affect Kir channel K^+ conductance as well.³⁶

1.4 KIR CHANNELOPATHIES

As already indicated, multiple human diseases are linked to mutations in inwardly rectifying potassium channels.³⁵ These disorders are a consequence of electrophysiological disbalance along with irregularities in potassium homeostasis. Anomalies in ion channel trafficking are largely responsible for Kir channel mutations, of which in most cases decreased forward trafficking takes place.³⁵ Even though mutations linked to trafficking can be found all over the primary sequence, they cluster in cytoplasmic domains. In regions playing a key role in gating, another collective of mutations is located, assumingly involved in the stability and folding of proteins.³⁵

Loss of function mutations in the ROMK gene, KCNJ1, result in type II Bartter syndrome, a rare autosomal recessive salt-wasting disease of the kidney. The main focus of this thesis lies on this nephropathy and therefore, Bartter syndrome type II will be extensively discussed in chapter 3. Andersen-Tawil syndrome is caused by a loss-of-function mutation in Kir2.1, which includes cardiac arrhythmias such as biventricular tachycardia eventually accompanied by long QT, periodic paralysis of the skeletal muscles as well as bone malformation of the fingers and the face.⁴⁶ On the contrary, short QT-syndrome and atrial fibrillation, the most common type of heart arrhythmia,⁴⁷ results from Kir2.1 gain-of-function mutations.⁴⁸ A severe repercussion of hyperthyroidism is thyrotoxic hypokalemic periodic paralysis, a Kir2.6 loss-of-function mutation affiliated with hypokalemia as well as skeletal muscle paralysis.^{2,49}

Keppen-Lubinsky syndrome, an extremely rare congenital disorder associated with Kir3.2 gain-of-function mutations, is primarily defined by intellectual disability and developmental delay.⁵⁰ Additionally, the disease includes symptoms like hypertonia and hyperreflexia and is furthermore characterized by lipodystrophy, microcephaly, a narrow nasal bridge and enlarged eyes. Germline mutations in KCNJ5, the gene encoding Kir3.4 leads to familial hyperaldosteronism type 3 (FH3).⁵¹ The loss-of function mutation results in severe hypertension and low potassium levels, a consequence of the channels loss of selectivity for K⁺.³⁵ The most common form of FH3 presents with symptoms of extreme hyperaldosteronism along with therapy resistance leading to bilateral adrenalectomy.⁵¹

As the focus of this thesis does not lie on Kir channelopathies, simply an overview comprising the most prominent examples of diseases associated with Kir channel mutations was given. Nevertheless, the listing demonstrates the crucial role inward rectifying potassium channels play in various mechanisms regarding function as well as regulation of the human body.

2 THE RENAL OUTER MEDULLARY POTASSIUM CHANNEL

2.1 INTRODUCTION TO ROMK

In humans, Kir1.1 is encoded by KCNJ1 and is more commonly referred to as ROMK: renal outer medullary K⁺ channel.³³ Initially referred to as ROMK1, the rat outer medullary K⁺ channel, ROMK was the first inward rectifier family member to be identified in the kidney as well as the first Kir family member to ever be cloned.^{39,8} In 1993 Ho and Herbert discovered an inwardly rectifying ATP-regulated potassium channel, differing from the, up to this point, typical voltage-dependent potassium channels in channel architecture and mechanisms of function.³⁹ The discovery of ROMK and its functional properties paved the way for a better understanding of renal physiology and nephrology.³³ Moreover, its finding led to the revelation of an entirely new potassium channel family.

The renal outer medullary K⁺ channel can be classified as an ATP-regulated or K⁺ transport channel with weak inward rectification.⁵² Along multiple other Kir channels, Kir1.1 is a tetrameric channel⁵³ that does not exist as a heteromer along with members of other Kir subfamilies.² ROMK is expressed in the kidney and brain. Although its role in the central nervous system remains relatively unknown, recent studies suggested an involvement of ROMK in mental diseases.⁵⁴ In the kidney however, Kir1.1 can be considered properly characterized and validated, given that its location and functional properties are well established. ROMK's involvement in potassium secretion in the cortical collecting duct (CCD) as well as its role in potassium recycling in the thick ascending limb (TAL) make the channel a key player in the regulation of potassium and salt homeostasis.³³

ROMK's clinical significance in human health and disease was established through identification of the genetic basis of Bartter syndrome type II, a rare autosomal recessive salt-wasting nephropathy caused by loss-of-function mutations in ROMK.³³ The tubulopathy presents with hyperaldosteronism, hyperreninemia along with hypokalemic metabolic alkalosis.⁵⁵

2.2 THE SIGNIFICANT ROLE OF ROMK IN THE KIDNEY

As its name indicates, the renal outer medullary K⁺ channel is mainly known for its crucial role in the regulation of kidney functions. The kidney is in charge of managing concentrations of various ions as well as extracellular fluid volume. In order to make this possible multiple ion and water carrier systems cooperate in the apical and basolateral membranes of renal epithelial cells.²⁷ Potassium channels fulfill essential tasks in maintaining blood and urine homeostasis by regulating levels of ions, such as Na⁺, K⁺ and Cl⁻. ROMK is considered a key player in those tasks. The channel is broadly expressed along the nephron and was localized especially at the apical membrane of renal epithelial cells.⁵⁶ ROMK can be found in epithelial cells of the cortical collecting duct (CCD), the connecting tubule (CNT),⁵⁶ the distal convoluted tubule (DCT) and the thick ascending limb of the loop of Henle (TAL).^{27,57} Via research on knockout mice and patch-clamp single channel recording it was established that Kir1.1 builds potassium secreting 35-pS K⁺ channels in principal cells of the CCD as well as 35-pS and 70-pS K⁺ channels at the apical membrane of TAL cells.^{27,28,29}

The Na⁺-K⁺-2Cl⁻ co-transporter, commonly referred to as the NKCC, is located on the apical membrane.³³ NKCC enables cells in the thick ascending limb of the loop of Henle to reabsorb a fourth of the filtered urinal Na⁺.⁸ Kir1.1 channels provide the Na⁺-K⁺-2Cl⁻ co-transporter with extracellular potassium, consequently NKCC is able to transport Na⁺, K⁺ and Cl⁻ into the cell. Therefore, ROMK keeps the co-transporter going, through what is called “K⁺ recycling”.^{8,27,58}

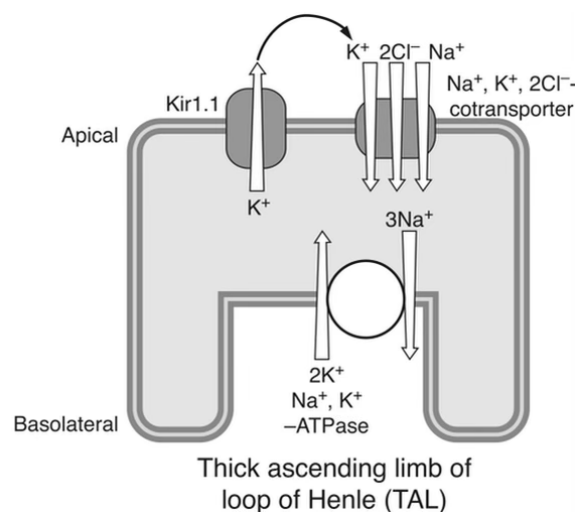


FIGURE 8: K⁺ RECYCLING IN THE TAL⁸

In the apical membrane of TAL cells the Kir1.1 channel is located closely to the Na⁺-K⁺-2Cl⁻ co-transporter, which enables their collaboration on ion transport in and out of cells.

Additionally, NaCl uptake is promoted as a result of NKCC's Cl^- absorption stimulated by ROMK.⁸ The Na^+/K^+ -ATPase at the basolateral membrane transports Na^+ accumulated in the cytoplasmic region to the extracellular fluid.⁸ ATPase activity is maintained by stimulation of unidirectional Na^+ transport from the apical to the basolateral side, facilitated through collaboration of ROMK with the $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ co-transporter.⁸ Moreover, the cell membrane potential (E_m) of TAL cells is hyperpolarized by ROMK, stimulating Cl^- exit via Cl^- channels from the basolateral membrane.²⁷ As a consequence of chloride ions exiting the tubule down its concentration gradient, the "lumen-positive transepithelial potential", which is the determining factor in paracellular transport of calcium, magnesium and sodium ions from lumen to the blood vessel side, is established.²⁶

The Cl^- channel CFTR, an abbreviation for cystic fibrosis transmembrane conductance regulator, is functionally coupled to ROMK on the apical membrane of TAL epithelial cells.⁸ Through CFTR's association with ROMK, the K^+ channel's activity decreases.⁸ It can be assumed, that interactions with NHERFs, the PDZ-domain binding protein, are responsible for this effect.⁵⁹ Activation of protein kinase A abolishes these interactions.⁶⁰ The apical K^+ conductance of TAL is enhanced by the antidiuretic hormone arginine vasopressin (AVP), ROMK's association with CFTR might contribute to this effect.⁸ The stimulation of ROMK by this hormone leads to a restriction of potassium secretion and urinary potassium loss.^{2,61} Furthermore, ROMK plays a central role in ion transport in cells of the cortical collecting duct, which is essential for potassium secretion into the urine.⁶²

2.3 ROMK IN THE BRAIN

Although high levels of ROMK expression in neurons of the cortex as well as the hippocampus were established via northern blotting and *in situ* hybridization in rat brain almost 30 years ago, the channels role in the central nervous system is still relatively unknown.^{8,63} In fact, the role of K⁺ channels in the brain in general remains a mystery for the most part.⁵⁴ However, several studies indicate an involvement of ROMK in mental diseases. In the striatum including the nucleus accumbens, an increase in ROMK mRNA expression in patients with schizophrenia and major depression was established.⁶⁴ Moreover, the ROMK gene KCNJ1 was associated with the personality trait of openness in a meta-analysis of a genome-wide study.⁶⁵ Further indication for ROMK's involvement in mood disorders, specifically its link to depression, is provided by research on the European honey bee venom peptide, Tertiapin (TPN), which selectively blocks ROMK.⁵⁴ In mouse behavioral tests antidepressive and anxiogenic effects were achieved through ROMK channel obstruction via TPN-RQ injection.⁵⁴

Additionally, it is worth mentioning that abnormal mental behavior was observed in patients with type II Bartter syndrome, a disease caused by deficiencies of the ROMK gene. Nevertheless it is unclear if the psychotic and mania-like symptoms stem from ROMK dysfunction in the brain or changes in the serum ion composition.^{66,67}

2.4 ROMK ISOFORMS

Despite the fact that six alternatively spliced isoforms of ROMK were distinguished, them being labeled Kir1.1a-f or ROMK1-6, only three of those isoforms, ROMK1, ROMK2 and ROMK3, also known as Kir1.1a, Kir1.1b and Kir1.1c, vary in sequence and length at the amino terminus.⁸ Exclusively the mRNA translation of ROMK1 and ROMK3 leads to different proteins as the other four isoforms.⁶⁸ ROMK3 contains the longest N-terminal sequence being 7aa longer than ROMK1 and 26 aa longer than ROMK2.² Thus, ROMK2 misses the 19 amino acid long sequence of ROMK1, which includes two putative PKC phosphorylation sites at S4 and T17. ROMK3 lacks both of those protein kinase C phosphorylation sites, yet it has one potential phosphorylation residue at T24.⁶⁹ The other putative PKC phosphorylation sites however, are identical in all three ROMK isoforms, along with the three protein kinase A phosphorylation sites.⁷⁰

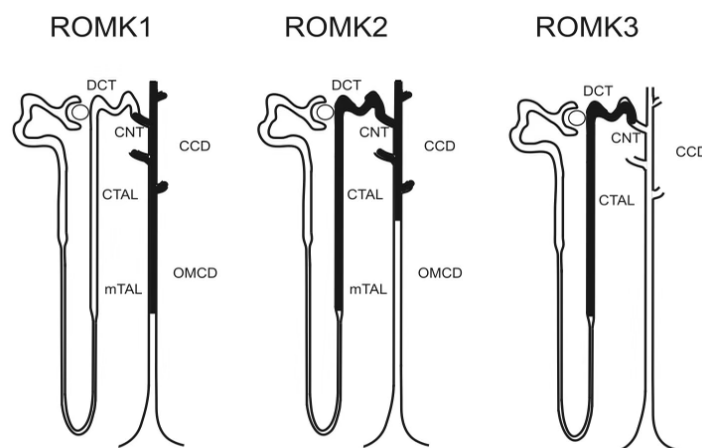


FIGURE 9: DISTRIBUTION OF ROMK ISOFORMS ALONG THE NEPHRON³³

Expression of ROMK isoforms in the kidney varies. ROMK1 can be found in the CNT, CCD and OMCD whilst ROMK2 is expressed in the TAL, DCT, CNT and CCD. ROMK3 transcripts are located in the TAL, DCT and CNT. Abbreviations: DCT: distal convoluted tubule, CNT: connecting tubule, CCD: cortical collecting duct, cTAL: cortical thick ascending limb, mTAL: medullary thick ascending limb, OMCD: outer medullary collecting duct.³³

In the kidney, ROMK isoforms are comprehensively represented and can predominantly be found in the renal cortex and outer medulla.⁶⁸ The expression of ROMK isoforms along the nephron varies as the splice variants carry out different physiological tasks.³³ Cells of the outer medullary collecting duct (OMCD) express solely ROMK1 transcripts whilst principal cells in the CCD express ROMK1 as well as ROMK2.⁵⁷ All three isoforms can be found in the CNT, where they regulate potassium transport.⁶⁸ Additionally, ROMK2 and ROMK3 transcripts are expressed by the TAL and DCT.⁷¹ In

the distal nephron, ROMK1 and ROMK3 are expressed exclusively in principal cells where they uphold potassium balance by managing K^+ secretion.^{33,62} In the thick ascending limb, shortened TAL, ROMK2 features to Na^+ reabsorption by driving Na^+ transport through $Na^+K^+-2Cl^-$ co-transporter activity.³³ Furthermore ROMK2 is responsible for apical membrane conductance.⁵²

2.4.1 PHOSPHORYLATION BY PKC VARIES IN ROMK ISOFORMS

To this day, it is commonly believed that the ROMK isoforms differ in their amount of serine phosphorylation sites. Although ROMK1 is in possession of three serine phosphorylation sites, serine residues 4, 183 and 201, residue 183 is not a PKC phosphorylation site.⁷² ROMK2 and ROMK3 however, only have two serine phosphorylation sites, which are located in the carboxyl-terminus.³⁹ Apart from the great importance PKC holds in ROMK surface expression, which will be further discussed down below, it fulfills multiple other functions in the regulation of this channel. Phosphorylation induced by Protein kinase C inhibits ROMK activity.^{73,69}

2.5 ROMK REGULATION & INTRACELLULAR LOCALIZATION

ROMK channel function and expression are controlled by various different factors such as phosphorylation by numerous kinases, both cytosolic and extracellular ATP, intracellular pH^{8,27} and post-translational modifications like ubiquitination, which is an enzymatic process of bonding an ubiquitin protein to a substrate protein.²

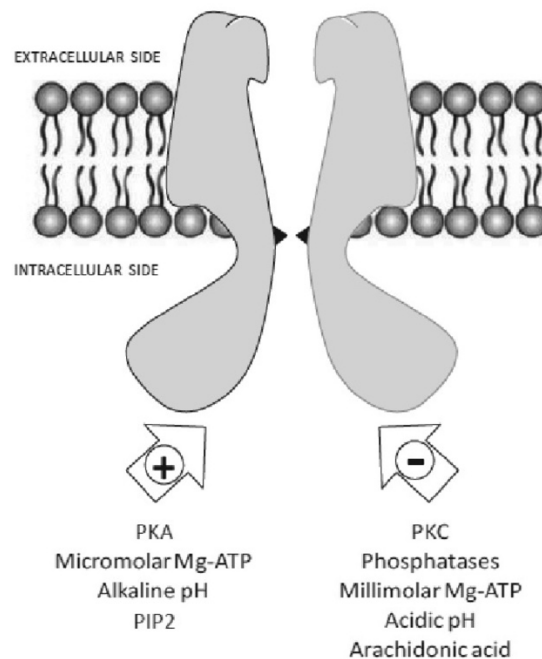


FIGURE 10: ROMK'S REGULATORY FACTORS⁷⁴

Activation and inhibition of ROMK are determined by the channel's main regulatory factors.⁷⁴

MicroRNAs, more commonly referred to as miRNAs, play a significant role in regulating the function of ROMK channels as well.² They are small single stranded non-coding RNA molecules responsible for post-transcriptional regulation of gene expression and RNA silencing. Studies have shown that the consumption of a considerable amount of potassium rich foods leads to an upregulation of multiple miRNAs which consecutively induces change in ROMK activity.⁷⁵ The activity of the protein Caveolin-1, which restricts the expression of ROMK at the plasma membrane, is decreased by miR-802, causing enhanced channel activity and surface expression of the channel.⁷⁵ Intersectin1 and the serine-threonine protein kinase WNK, an abbreviation for “with no lysine (K)”, reduce cell surface expression of ROMK. miR-194 counteracts this regulation, therefore enhancing ROMK surface expression.⁷⁶

Furthermore, ROMK is regulated by lipids, such as endogenous phospholipids and arachidonic acid.⁷⁴ AA inhibits ROMK channel activity, even though other associated fatty acids have no influence on the potassium channel.^{69,77} Specifically the ROMK1 splice variant is affected, however, arachidonic acid has less impact on other ROMK isoforms, presumably due to PKC-mediated phosphorylation of ROMK1.^{72,77} ROMK's regulation by Mg-ATP depends on the concentration of the nucleotide.⁷⁴ On the one hand, PKA's effect on ROMK is improved by low intracellular Mg-ATP concentrations in micromolar range. On the other hand, Mg-ATP functions as a ROMK inhibiting factor in higher millimolar concentrations.⁷⁸ Another ROMK inhibitor is cholesterol.² Site-directed mutagenesis and modeling studies indicate that the ROMK-cholesterol interaction residues can be found between α -helices of neighboring channel subunits.⁷⁹ Moreover, it was established that these interactions include aromatic as well as hydrophobic residues.

Located in the COOH terminus, ROMK has ER retention signals: 368R-X/A-R370 and K370-R371.^{2,80} Multiple intracellular protein kinases like PKA and PKC regulate the trafficking of ROMK to the cell surface via post-translational modification.⁸ Consequently, through promotion or suppression of ROMK surface expression, physiological signaling pathways manage contribution to the channels current.⁸

2.5.1 SURFACE EXPRESSION SUPPRESSORS

2.5.1.1 UBIQUITINATION

Ubiquitination negatively regulates ROMK as it decreases the surface expression of channel protein.⁸ K22, also referred to as Lys-22, is located in the amino terminus of the channel and its monoubiquitination causes the channels lysosomal degradation.⁸¹ As a consequence, preventing this post-translational modification increases surface expression of ROMK. The mutation from lysine to arginine results in a significant rise of ROMK channel activity.⁸

2.5.1.2 WNKs

Members of the WNK family contribute to ROMK's task at the apical plasma membrane and play a key role in maintaining the balance between NaCl absorption and potassium

secretion.⁸² Primarily expressed along the aldosterone-sensitive distal nephrons (AS-DNs), the With-no-lysine kinases WNK1, WNK3 and WNK4 are of fundamental importance in ROMK regulation.^{82,83} The WNK1 gene produces two main isoforms: long WNK1 (L-WNK1), which is catalytically active as well as omnipresent and its kidney-specific kinase-deficient shorter isoform KS-WNK1.⁸⁴ Pseudohypoaldosteronism type II (PHAII), a thiazide-sensitive, chloride-dependent inherited syndrome associated with hypertension and hyperkalemia is attributed to mutations in the WNK1 and WNK4 genes.^{85,86} A switch in WNK1 isoform along with liberation from WNK1 inhibition by KS-WNK1 is required for a physiologic up-regulation of ROMK activity, since the loading of dietary K⁺ boosts the relative abundance of KS-WNK1 to L-WNK1 protein and transcript in the kidney.⁸⁶ KS-WNK1 is unable to block ROMK since it has no kinase activity, nevertheless KS-WNK1 happens to activate ROMK indirectly through blockage of L-WNK1.^{83,86} Clathrin-dependent endocytosis of ROMK via WNK4 suppresses the channel, a mechanism independent of WNK4 kinase activity.⁸⁷ Although WNK1 affects ROMK surface expression in a similar way, it does so independently of WNK4. WNK1 speeds up endocytosis, thereby reducing ROMK surface expression.⁸⁸ WNK3 decreases ROMK expression at the plasma membrane, therefore inhibiting channel activity. Equally to ROMK's blockage by WNK4, WNK3's kinase activity is not required for this effect.⁸⁹

Intersectin is an endocytic scaffold protein that interacts with both kinases, WNK1 and WNK4.⁶⁸ WNKs induce ROMK1 endocytosis through these interactions.⁹⁰ Via surface biotinylation studies in COS-7 cells it was shown that the modular clathrin adaptor protein ARH (autosomal recessive hypercholesterolemia) facilitates ROMK endocytosis in an NPNF-dependent manner, meaning the NPNF motif is a necessity for sufficient ARH-ROMK interaction.⁹¹ For endocytosis stimulated by WNK1, ARH recruits ROMK to clathrin-coated pits. Moreover, a rare inherited form of hypercholesterolemia is caused if ROMK binds directly to ARH.⁶⁸ Through increase of ROMK endocytosis from the apical plasma membrane, the channel can be inhibited.⁶⁸ Insulin and IGF-1, the insulin growth factor, belong to the PI-3 kinase activating hormones, which through phosphorylation of WNK1 in a Akt1/SGK1-dependent manner achieve this inhibiting effect on ROMK.⁹²

2.5.2 SURFACE EXPRESSION ENHANCERS

2.5.2.1 SGK1

The serum-glucocorticoid-regulated kinase is an aldosterone-induced serine/threonine protein kinase that, as shown by the usage of ROMK1 or ROMK2 in cell-based experiments, modifies ROMK expression and function via three different mechanisms.⁹³ Firstly, SGK1 phosphorylates the serine residue S44, located at the NH2 terminus of ROMK, leading to increased surface expression as well as enhancement of current, an effect further in dependence of the combined action of SGK1 with NHERF2, the Na⁺/H⁺ exchange regulating factor.^{94,95} Secondly, a shift in pH sensitivity of ROMK1 to more acidic values is achieved by phosphorylation of S44, which at cytosolic pH 6.6-7.3, increases electrophysiological function.⁹³ Third, clathrin-dependent endocytosis of ROMK2 can be prevented via phosphorylation of serine residues 1169 and 1196 on WNK4 by SGK1, causing an increase in ROMK2 plasma membrane expression.⁹³

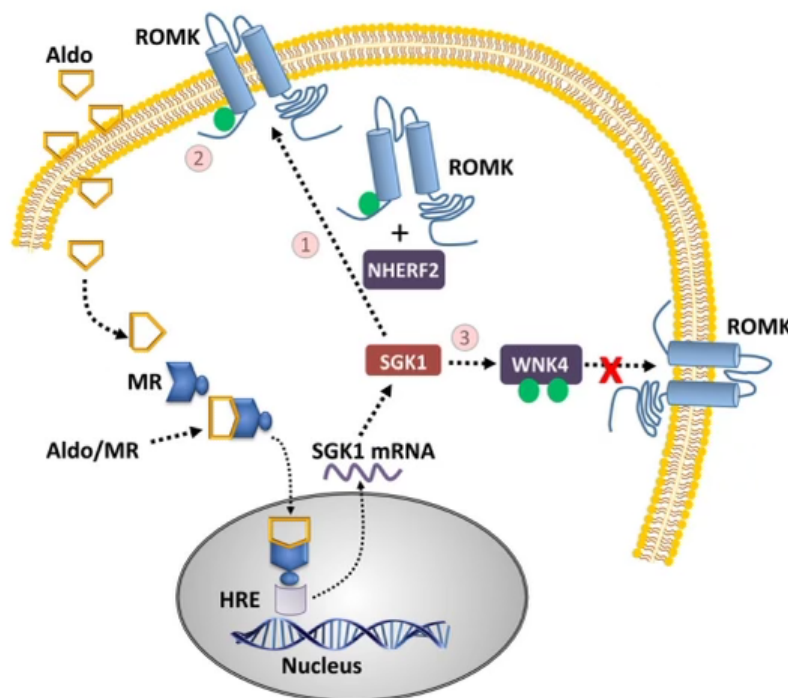


FIGURE 11: REGULATION OF ROMK VIA SGK1⁹³

After the aldosterone/MR (cytosolic mineralocorticoid receptor) complex promotes transcription of SGK1 in the nucleus, ROMK activity is up-regulated by SGK1 via three different mechanisms: 1) Direct phosphorylation of ROMK at S44 leads to enhancement of ROMK trafficking via NHERF2. 2) Increased electrophysiological channel function by direct phosphorylation of S44. 3) Bi-phosphorylation of WNK4 results in reduction of clathrin-dependent endocytosis of ROMK.⁹³

2.5.2.2 PKA

Protein kinase A, often referred to as PKA, is a cAMP dependent protein kinase belonging to the serine/threonine kinase family. ROMK has three PKA phosphorylation sites.³³ S44 is located in the amino-terminus of the channel whereas the other two phosphorylation sites, S219 and S313, can be found in the carboxyl-terminus.⁸ More precisely, S219 is located at the peak of the cytoplasmic domain, favorably positioned in between an intersubunit salt bridge, reaching from R311 to E302, the G-loop and a PIP2 binding site.³³ Surrounded by the intrasubunit (R41-E318) and the previously mentioned intersubunit salt bridges, the residue S313 can be found on the cytoplasmic domain surface.³³ Phosphorylation of S44 results in suppression of the ER retention signal, allowing ROMK delivery to the cell surface.⁹⁶ Therefore, ROMK trafficking to the plasma membrane is boosted via phosphorylation of S44 by either PKA or SGK.^{70,97,98} Channel activity is eliminated, if any two of the three previously mentioned phosphorylation sites are mutated.⁷⁰ ROMK's channel open probability is increased via PKA-mediated phosphorylation of S219 and S313, likely by enhancement of interaction between PIP2 and ROMK.^{99,100} The proximity of these two serine residues with PIP2 and pH gating determinants enables efficient function coupling between pH-dependent gating, PIP2 interaction with the channel and PKA phosphorylation.³³

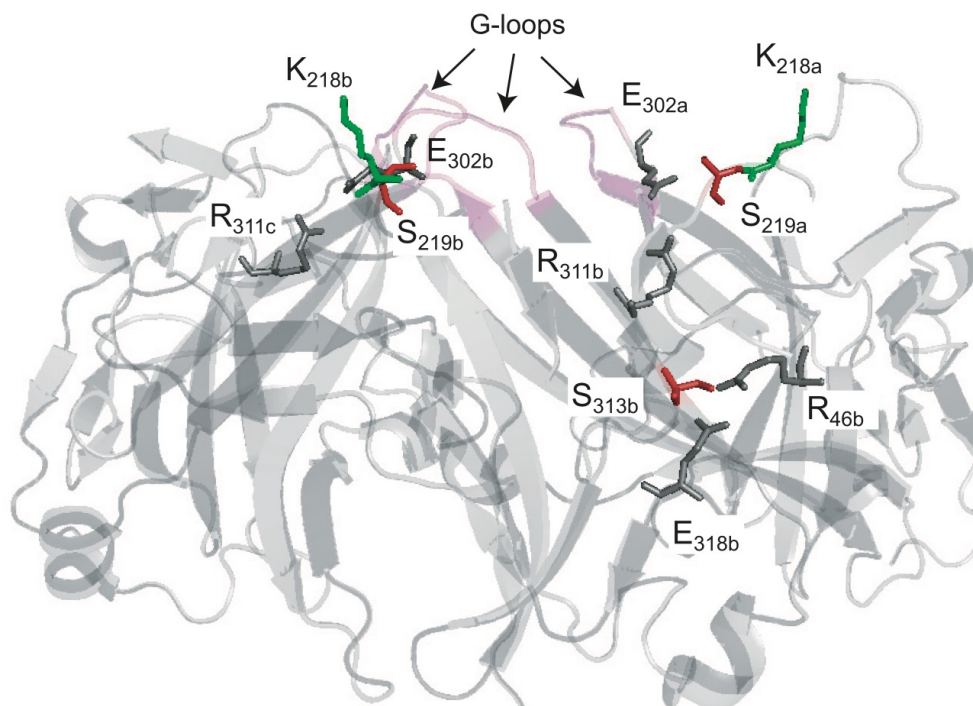


FIGURE 12: IMPORTANT ROMK RESIDUES³³

The PKA phosphorylation sites S219 and S313 are marked red. The intrasubunit (R41-E318) and intersubunit salt bridges (R311-E302) are displayed. In order to classify the residues into subunits, alphabetic labeling a,b,c or d was used. The cytoplasmic domains of 3 subunits are displayed. The PIP2 binding site K218 is colored green and the G-loops are marked magenta.³³

If ROMK is phosphorylated by PKA, the channels affinity for PIP₂ is enhanced.¹⁰¹ Therefore, mutation of residues usually phosphorylated by Protein kinase A, leads to a decrease in ROMK's affinity for Phosphatidylinositol 4,5-bisphosphate. Direct communication of ROMK with PIP₂ is necessary for channel opening.¹⁰¹ In the cortical collecting duct, the two protein kinases PKA and PKC play a significant role in the regulation of apical potassium channels in general, as they are responsible for their activation as well as their inhibition.¹⁰¹ It should be noted that Bartter's syndrome can be caused by mutation of a PKA phosphorylation site.¹⁰²

2.5.2.3 PKC

Protein kinase C, commonly abbreviated to PKC, is a multi-functional protein kinase with various isoforms and is, like Protein kinase A, a member of the serine/threonine kinase family. For the surface expression of ROMK, the phosphorylation of its two serine residues, S4 and S201 by protein kinase C is a necessity.⁷² It was established by Biotin labeling technique in human embryonic kidney (HEK) cells, that PKC blockage decreases ROMK surface expression.⁷² In *Xenopus* oocytes it was demonstrated via confocal microscope imaging that mutations of those serine residues decrease surface expression as well.⁷² Therefore, PKC acts as a surface expression enhancer in HEK cells and *Xenopus* oocytes. PIP₂ weakens PKCs ability to block ROMK.¹⁰¹ Through charge-charge interaction PKC moves ROMK pH sensitivity in the direction of more neutral pH levels¹⁰³, which makes the channel less sensible to PIP₂.¹⁰⁴

2.6 STRUCTURE-FUNCTION RELATIONSHIPS

To this day, the structure of the renal outer medullary potassium channel has not been fully resolved yet. Since the prokaryotic KirBac1.1 channel is a homologue of eukaryotic Kir channels, its crystal structure has been heavily utilized for the creation of eukaryotic in silico Kir channel models,¹⁰⁵ therefore helping to better understand the structure of ROMK.⁷⁴ Like all other members of the inward rectifier potassium channel family, ROMK consists of the two transmembrane domains TM1 and TM2, linked by a pore-forming P-loop sequence, which contains the potassium selectivity filter.^{33,106} Both, the amino as well as the carboxy terminal domain are located in the inner membrane side, building a distinctive cytoplasmic extended pore structure.¹⁰⁷ Functioning ROMK homotetramers are created by four associated subunits.³⁶ Even though most members of the Kir channel family are able to form heterotetramers⁸, for ROMK and Kir7.1 only the building of homotetramers is possible.^{53,36} The cytoplasmic domains serve as binding sites for several regulatory factors such as lipids, nucleotides and pH. Moreover, phosphorylation and dephosphorylation takes place there.⁷⁴

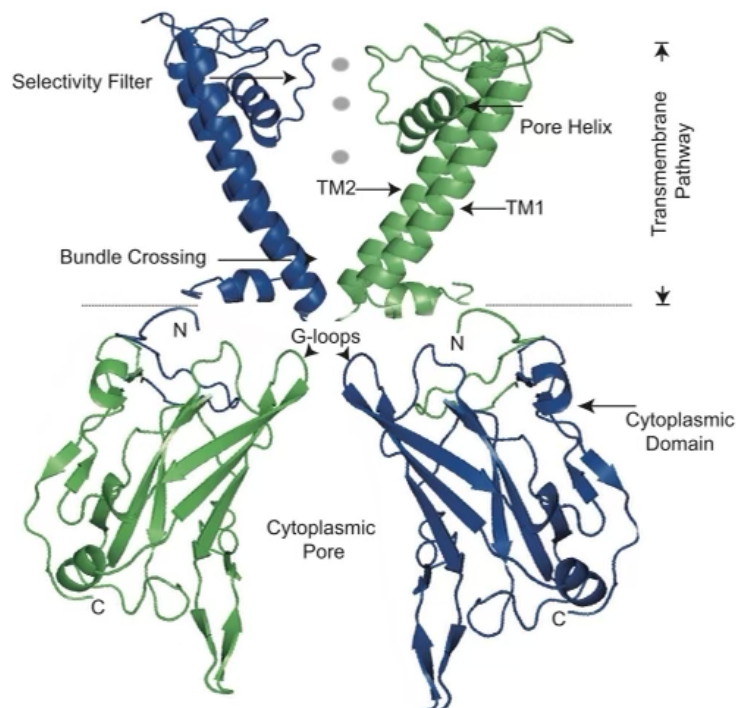


FIGURE 13: ATOMIC ROMK MODEL³³

An atomic ROMK model was created with known Kir channel structures and comparative protein structure modeling.¹⁰⁸ Displayed are residues 34-356 of ROMK1. Residues 60-64 as well as 185-191 are not resolved yet. PyMol software was used for image rendering. TM1, TM2 and the first and second transmembrane domains are exhibited.³³

2.6.1 POTASSIUM SELECTIVITY

The central ROMK pore, passing through the plasmalemma, is enclosed by four subunits.³³ The P-loop is the tightest area of the open pore conduction pathway. It is formed by linkers, reaching from the extracellular space into the membrane, thereby connecting the two transmembrane domains.³³ In the P-loop the conserved potassium selectivity sequence, shaped into a strand conformation and characterized by a “T[V/I]GYG” motif, can be found.¹⁰⁹ Showing to the pore are the carbonyl oxygens of the sequence’s backbone. Four potassium binding sites, which are equal in distance from each other, are built by the stringing together of equivalent structures from the four ROMK subunits, a feature that all potassium channels have in common.³³ At every binding site one potassium ion is enclosed by four oxygen atoms. Diffusion of potassium out of the aqueous environment is facilitated by the selectivity filter, as it imitates the inner hydration shell of potassium, therefore making it, from an energetic standpoint, beneficial to leave.³³ The binding sites do not compensate dehydration energy of smaller ions, like sodium, consequently being potassium selective.¹¹⁰ ROMK has a significantly high selectivity for potassium: $P_{K/Na} > 20 - 100$ ($P_{Ti} > P_K > P_{Rb} > P_{NH4}$)^{33,111}

2.6.2 POTASSIUM CONDUCTION

Biophysical measurements revealed that by means of a multiple ion conduction mechanism potassium travels through the ROMK pore.¹¹² Depending on the selectivity filter’s conformation, pairs of K^+ ions occupy various of its binding sites at the same time, as proven in the archetypical KcsA channel.^{33,113} The two potassium ions, which are present in the filter at the same time, cause electrostatic repulsion.¹¹⁰ In order to acquire a conductive filter that contains two potassium ions, the filter needs to change its conformation, which in combination with the potassium affinity lowering effect of electrostatic repulsion, leads to high conduction rates.¹¹⁰ Facilitated via a highly effective cycle of K^+ entry, binding, change in conformation, combined with electrostatic repulsion, potassium travels through the pore with amazing selectivity as well as outstanding throughput ($\sim 10^8$ K^+ ions/s).^{33,110}

Even though the selectivity filter is a highly conserved motif in potassium channels, they differ in their K^+ binding rates, which probably leads to single-channel conductance dissimilarities.³³ Since the P-loop forms the selectivity filter and potassium binds

at the selectivity filter, the P-loop plays an important role in ROMK's single-channel conductance.¹¹⁴ Consequently, it can be assumed that P-loop residues, which regulate the filter's flexibility and structure, have an impact on variations in single-channel conductance.³³ The highly conserved glutamate-arginine residue pair E136-R147 of ROMK's P loop is responsible for maintaining the fixed bowl-like structure displayed by the selectivity filter.^{33,115} The residue V140 can be found between the pore helix and the selectivity filter. It is known to affect barium block as well as cation selectivity.³³ Additionally, variations in single-channel conductance between ROMK and Kir2.1 are partially attributable to V140.¹¹⁶

2.6.3 RAPID GATING

The shift between the shortest-lived closed (~ 1 ms) and the open (~ 10 ms) state happens quickly, making ROMK a fast gating channel.^{33,117} Potassium concentration determines how fast ROMK enters the short-lived closed state. Additionally, this rate is proportional to the intensity of ROMK current.³³ As a consequence, it was assumed that potassium permeation is a crucial factor in ROMK's rapid gating process.¹¹⁷ It was established through molecular simulation studies that the structure of the selectivity filter depends on whether and where there is potassium in the pore.³³ If a certain position in the selectivity filter is occupied by potassium, conformation of T141-I142 changes rapidly, causing the channel to shift into a nonconducting conformation.^{33,118} Simultaneously fast closure happens, indicating a fast gating mechanism.³³

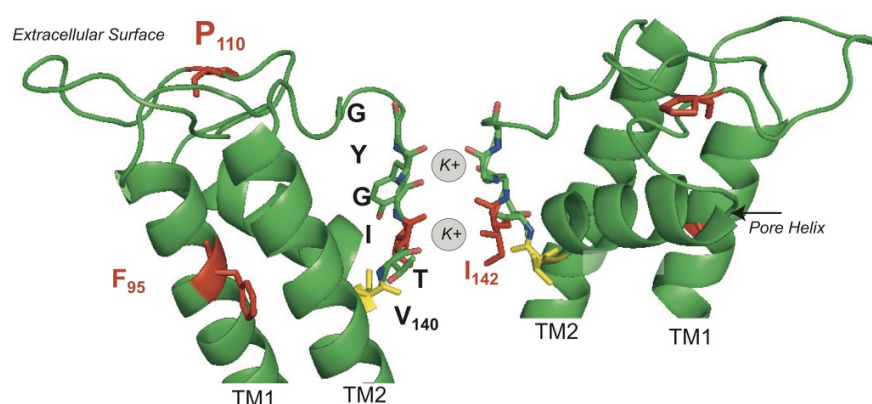


FIGURE 14: THE POTASSIUM SELECTIVITY FILTER³³

The potassium selectivity sequence is characterized by the "T₁₄₁IGYG" motif and shaped into a strand conformation. Its backbone carbonyl oxygens, marked in red, show into the pore, thereby imitating the K⁺ hydration shell. Between the pore helix and the selectivity filter the residue V140 can be found, playing a significant role in ROMK barium block and single-channel conductance. ROMK's fast gating process is supported by a rapid change in conformation of T141-I142. In Bartter syndrome type II the red marked residues are mutated.³³

2.6.4 INWARD RECTIFICATION

As previously mentioned, the phenomenon of inward rectification is a mechanism that all Kir channels have in common. At, relative to the resting membrane potential, positive potentials, magnesium ions^{119,120} and polyamines⁴⁴ enter the cytoplasmic pore and bind to a location near the selectivity filter which prevents further potassium permeation.¹²¹ However, the intensity of inward rectification is distinct between members of the Kir channel family, considering the difference in affinity for polyamines and Mg^{2+} and varieties in binding site.³³

The uncharged Asparagine (Asn, N) N171 in TM2, the second transmembrane domain, is responsible for the weak inward rectification displayed by ROMK.⁸ By replacing the Asparagine with Aspartic acid (Asp, D), the affinity for magnesium ions can be increased and strong rectification is achieved.^{119,122} As a consequence, the residue is commonly referred to as the “D/N site”.⁸ ROMK exhibits strong inward rectification when the N171D mutation occurs and contrary, the D172N mutation in the usually strong inward-rectifying Kir2.1 channel causes weak inward rectification.¹²² The side chain of the residue N171 reaches from TM2 into the pore, which is in line with a transmembrane potassium conduction pathway binding site.³³

2.6.5 REGULATION OF GATING

The gate regulated by PIP2, ATP and cytoplasmic alkalization can be found in the transmembrane conduction pathway.³³ The Kir channel gating mechanism is not fully understood and much of it remains a mystery. Nevertheless, the idea that departure of TM2 from the bundle crossing region plays a role in regulated channel opening, is compliant with ROMK mutagenesis studies and structural homology modeling.³³ The glycine residues G167 and G176 play a crucial role in the regulation of ROMK's gating mechanism.¹²³ In their function as flexible hinges, these glycines make it possible that the TM2 helices can move away from each other.¹²³ Assuming this hypothesis is correct, the hinge-dependent movement would transport the putative gate at TM2's cytoplasmic end away from the pore.³³ G167 and G176 will be further discussed down below in the chapter 2.8.3.3 “Residues in the TM2 transmembrane region”.

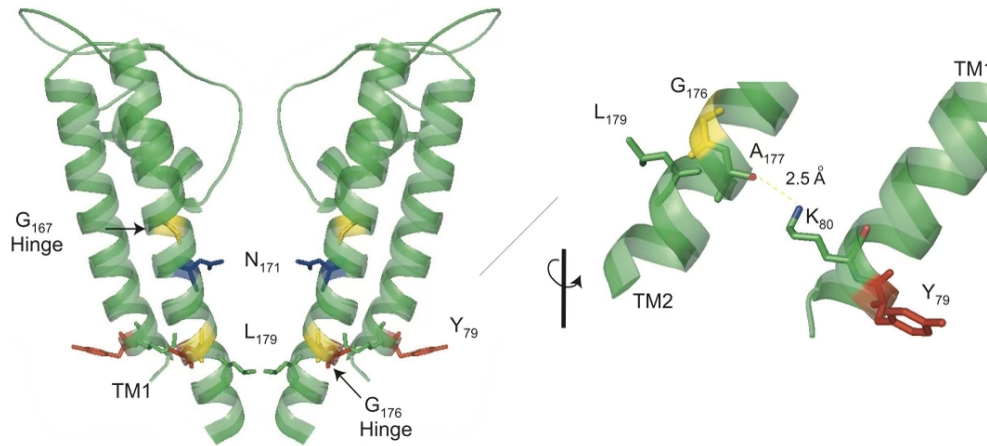


FIGURE 15: THE TRANSMEMBRANE POTASSIUM CONDUCTION PATHWAY³³

The crucial residues of regulated channel gating and inward rectification are located in ROMK's transmembrane potassium conduction pathway. The two glycine hinges G167 and G176 play a crucial role in ROMK's gating movement, allowing the TM2 helices to move away from each other, thereby transporting the putative gate, residue L179, out of the pore. The gate is stabilized by H-bonding between K80 at the TM1 base and the TM2 residue A177. In Bartter syndrome type II the red marked residue Y79 is mutated.³³

The hydrophobic alkane side chain of L179 reaches from the bottom of TM2 into the pore.³³ The open state is stabilized, if L179 is switched with small or highly polar side chains, corresponding with the idea that this residue operates as the regulated gate, obstructing the conduction pathway in the closed state.¹²⁴ Moreover, the gating process is regulated by interactions between the two transmembrane helices.³³ H-bonding between the ϵ -nitrogen of K80 and the backbone carbonyl oxygen of A177 plays a significant role in the pH- and PIP2-dependent gating process, as it stabilizes the gate.^{125,126}

2.7 THE CYTOPLASMIC DOMAIN

2.7.1 PORE EXTENSION

Approximately 30 Å far from the canonical transmembrane potassium conduction pathway, a long water-filled pore extends, built by assembled cytoplasmic ROMK regions.^{33,127} Carboxy-terminal beta strands, which are positioned in an antiparallel order, cover the inner cavity of the cytoplasmic pore with acidic residues.³³ The architecture of this arrangement is no coincidence, as established via electrostatic free energy calculations, the thereby created environment facilitates blocker binding by cations as well as efficient transport of K^+ .¹²⁸

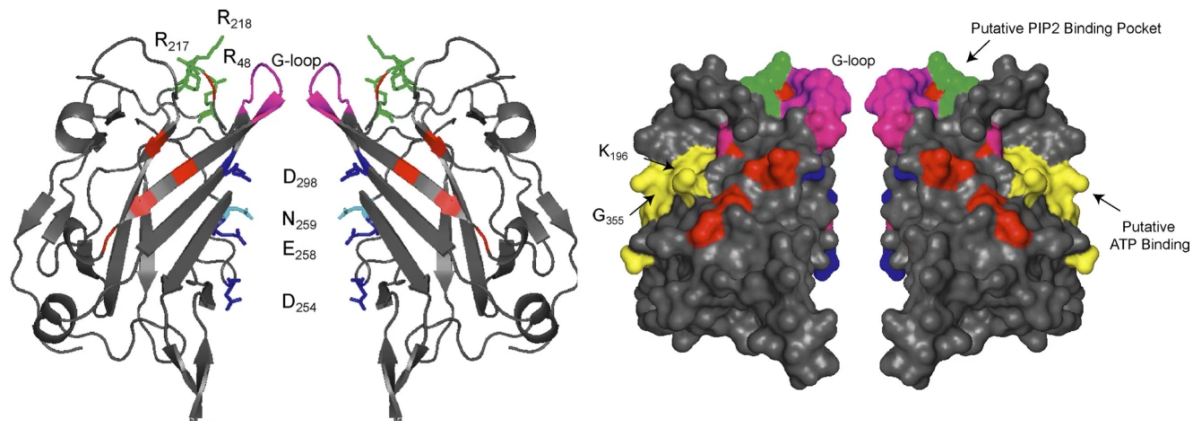


FIGURE 16: THE CYTOPLASMIC DOMAINS OF ROMK³³

The left image is in standard cartoon view whereas the right one is surface rendering. Marked in blue are the pore lining acidic residues. The residue N259 is displayed in light blue and is known to be a significant determinant of single-channel conductance. The G-loops at the peak of the cytoplasmic domain are marked in violet. Binding residues of PIP2 are displayed in green and marked in yellow are the putative ATP binding domains. Bartter syndrome mutations are colored red.³³

Interestingly, multiple residues involved in single-channel conductance and cation block match with residues participating in the creation of the favorable electrostatic field.³³ For instance, residues regulating the polyamine entry rate correspond to ROMK cytoplasmic pore amino acids: D254, E258, D298.¹²¹ Via the cytoplasmic pore, some inner wall residues function as direct potassium transport regulators by energetically blocking K^+ traffic.³³ Thus, unattached to selectivity filter residues, single channel conductance can be altered. The residue N259 is the most important single-channel conductance determinant, as its side chains covers the inner pore wall.^{33,129} Moreover, the residue strongly contributes to ROMK surface charge.¹²⁸

2.7.2 THE G-LOOP

Stemming from the carboxy-terminal β C- and β D-strands of every subunit are four identical loops.³³ Through fusion of those loops, a narrow opening at the cytoplasmic pore peak is built, the G-loop.³³ Next to the helix bundle crossing (HBC) gate, the G-loop exhibits another distinctive cytoplasmic gate in Kir channels.^{2,43} In between the cytoplasmic and the transmembrane pore, the G-loop probably generates a flexible diffusion barrier.³³ Inward rectification as well as channel gating is modified through G-loop residue mutations in Kir2.1 and Kir3.1, matching to the residue T300 in ROMK1.¹³⁰ It was shown through Kir2.1-ROMK chimeras, that the corresponding ROMK residues 294-320 take part in single channel conductance.³³ A revelation that corresponds with the hypothesis that the G-loop functions as a resistance barrier to ROMK ion flow.¹²⁹

GIRK channel crystal structures show two distinct conformations of the G-loop gate.¹⁰⁷ Via rigid body displacements of the subunits, the cytoplasmic pores adopt different conformations while the transmembrane pores stay the same.³³ The apex in one structure of the cytoplasmic domain (CTD) was dilated (or open), whereas in the other one it was constricted (or closed).¹⁰⁷ In dilated form, oxygen atoms are displayed on its surface, therefore a partially hydrated potassium can travel through the pore in between the G-loops, as they are sufficiently far away from each other.³³ Methionine sulfur atoms cover the constricted conformation.¹⁰⁷ In this shape, the conduction pathway is blocked and as a consequence, passage of even a smaller, dehydrated potassium is not possible.³³ Via state-dependent chemical modification studies it was proven, that during the pH-dependent gating process of ROMK, the G-loop residue C308 changes its conformation.¹³¹ Thus, exhibiting the physiological importance of these conformational changes.

Residues in or near the G-loop were defined as crucial factors in ligand-dependent gating through electrophysical studies as well as mutagenesis in various members of the Kir channel family, such as Kir2.1¹³⁰, Kir3.2¹³² and Kir6.2¹³³, indicating the fundamental significance of the G-loop in the Kir channel gating mechanism.³³ Further proof of the essential role the G-loop plays in inward rectifiers is the fact that mutations in residues affecting this structure lead to diseases in multiple Kir channel family members.^{33,107,134} For example, potassium conduction is obstructed by A306T, a Bartter syndrome mutation.¹³⁵

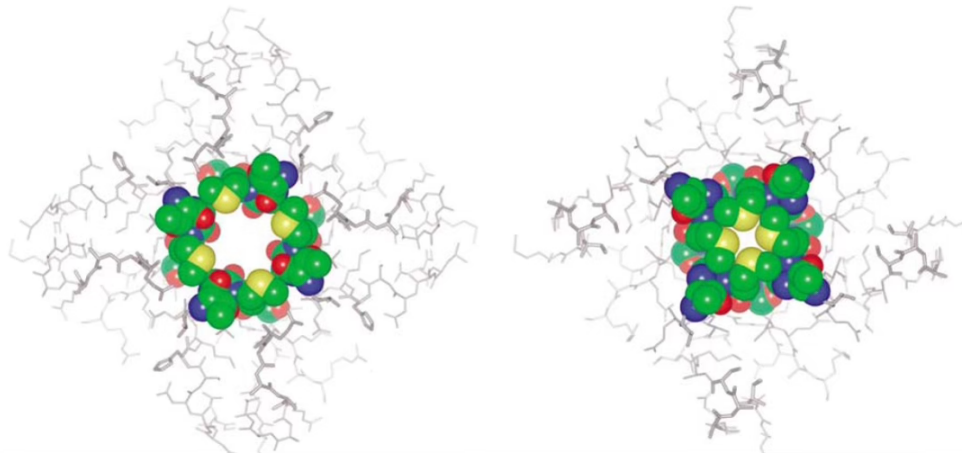


FIGURE 17: THE TWO G-LOOP CONFORMATIONS¹⁰⁷

Crystal structure of the GIRK channel. A view on the cytoplasmic pore from the transmembrane side. On the left, the apex of the dilated cytoplasmic pore is displayed and, on the right, its constricted conformation. The G-loop residues 306-309 are exhibited as a CPK model. Nitrogen atoms are marked in blue, oxygen in red, carbon in green and sulfur in yellow. Cytoplasmic pore regions around the apex are presented with a wire model. The scale bar is 10 Å.¹⁰⁷

Via conformational G-loop changes, phosphorylation at adjacent residues or binding of ligands, like PIP2 on the cytoplasmic domain surface could, bearing the approximation of the G-loop to the TM2 bundle crossing in mind, likely be allosterically linked to movement of TM2.^{33,130} Gating defects that are responsible for diseases in Kir2.1 as well as Kir6.2 and entail mutated residues of the G-loop, have been associated with coupling mechanism defects.¹³⁴ In ROMK however, further studies on this topic are essential.³³

2.8 MODULATION OF THE ROMK CHANNEL

2.8.1 ATP

In various studies the Steve Herbert's group provides evidence that ATP maintains a direct interaction with ROMK.³³ In order to establish to which part of the Kir channel ATP binds to, the scientists used the fluorescent ATP analogue 2',3'-O-(2,4,6- trinitro-phenylcyclo-hexadienylidene) adenosine 5'- triphosphate (TNP-ATP) and examined it's binding to maltose-binding fusion proteins of the cytosolic N-terminal and C-terminal regions of four different Kir channels (Kir1.1, Kir2.1, Kir6.1 and Kir6.2).¹³⁶ These experiments determined, that the COOH termini of the examined channels are not only sufficient, but necessary to bind TNP-ATP, whereas the NH2 termini are not able to bind TNP-ATP.¹³⁶ Further elaborated, for ATP to bind to ROMK1 with high affinity, the first 39 carboxy-terminal amino acid residues (183-221) are enough.^{136,137} Essential for successful ATP-ROMK interaction are the three arginines R188, R203 and R217, residues that are also responsible for PIP2-ROMK interactions.¹³⁸ Therefore, it can be assumed that these residues play an important role in the antagonistic ROMK regulation by ATP and PIP2.³³ Moreover, two distal ROMK carboxy-terminal segments, G335KY and F344GKTVE as well as the two residues K196 and R212 are crucial for ATP binding with high affinity.^{33,139} The importance of ATP was further exhibited in a series of Kir channel experiments in ATP-free solutions.¹⁴⁰ Run-down activity, meaning a channel activity decline, was observed by excising the Kir channel into inside-out patches in ATP-free solutions. Hydrolysable ATP as well as PIP2 types were able to rehabilitate the channel from run-down activity.^{2,140}

2.8.2 PIP2

PIP2 is a signaling phospholipid located in the inner leaflet of the plasma membrane and crucial for functioning Kir channel gating.^{2,8,140} PIP2 binds to the interface between the transmembrane domain and the cytoplasmic domain, where it assumingly induces a 6 Å shift of the cytoplasmic domain in the direction of the transmembrane domain.² Via highly conserved salt bridge interactions the negatively charged phosphate groups of PIP2 interact with the Kir channel.² There are a few basic amino acids that are absolutely critical for PIP2 activation of Kir channels:^{2,141} RWR, containing the residues R78 and R80, is a highly conserved sequence motif in many members of the inward

rectifier channel family.² In ROMK there is a lysine instead of an arginine at R78. At the amino terminus of the outer helix, the 1' phosphate of PIP2 interacts with this arginine and with R80.² 186-RPKKR-190 is a B-loop region sequence motif. The 4' and 5' phosphates interact with its highly conserved residues as well as with the completely conserved K183 residue of the transmembrane region TM2.²

Depending on the degree of stereospecificity and affinity to PIP2 over PI(3,4,5)P3 and PI(3,4)P2, the seven Kir subfamily members can be divided into four different groups⁴²: highest, moderate, weak or low affinity. ROMK belongs to the moderate affinity group². As established via systematic mutagenesis, normal PIP2 dependent gating in ROMK and in some of its fellow Kir channel family members requires multiple positively charged cytoplasmic amino acid residues, corresponding to R48, K181, K184, K186, R188, R217, K218, R311 in ROMK1.^{33,142} Channel activity is reduced through mutagenic neutralization of these residues. Moreover, exposing the Kir channel to polycationic chelating agents or anti-PIP2 antibodies results in channel inactivation, which is achieved faster through mutagenic neutralization.³³ In conclusion it can be stated, that via interaction with specific amino-acids of the carboxy-terminus, PIP2 activates ROMK, particularly the ROMK1 variant.^{74,142}

PIP2 has a negatively charged head group that interacts with positively charged amino acids. Identification of those channel surface residues indicates a conserved PIP2 interaction site.³³ MacGregor et al. established that the ROMK amino acid residues 183-221, including K181, K184, K186 and R188 form a carboxy terminal peptide that binds PIP2 directly.^{33,137} These revelations point more in the direction of a binding site rather than at an allosteric modulator. In Kir crystal structures, this part of the ROMK channel remains partially unresolved, but corresponds well to the original carboxy-terminus.³³ Located at the apex of the cytoplasmic domain, this COOH terminus stretches from the transmembrane domain TM2. Via mapping the other basic residues in the 3D model, it was determined that at the peak of the cytoplasmic domain, most of these residues, them being R48, R217 and R218, cluster, probably pointing to the inner lipid leaflet.³³ In summary, it can be stated, that those findings suggest that PIP2 attaches to the apical surface of the cytoplasmic domain directly, which corresponds well with the idea that the signaling phospholipid binds the extended cytoplasmic pore to the plasma membrane, consequently keeping the channel in an open state.³³

Various mutations in PIP2 residues lead to Kir channelopathies. Dysfunctional PIP2-dependent gating is displayed in multiple Bartter mutations, for example C49Y, I51T, A214V, and L220F.¹⁴³ Location of the residues was established via mapping on the 3D ROMK model.³³ They are gathered near the putative PIP2 binding site on the membrane-facing surface of the cytoplasmic domain. Therefore, the mutations probably cause PIP2 binding pocket changes.³³

2.8.3 PH SENSITIVITY

Even though intracellular pH controls several members of the Kir channel family, for instance Kir1.1, Kir2.3, Kir4.1, Kir5.1 and Kir6.2, Kir1.1 and Kir4.1 seem to react more sensitive to changes in pH than the other channels.¹⁴⁴ For example, intracellular acidification reduces channel activity of ROMK, as the channel closes with a pK_a of 6.5.^{145,146,147} Various residues in the renal outer medullary potassium channel are responsible for its pH sensitivity. Hibino et al. divide them in three groups: 1) R41, K80 and R311 are positively charged residues; 2) L160 in the bundle crossing region 3) residues in the transmembrane region TM2.⁸ It was originally assumed that R41, K80 and R311, the Arg-Lys-Arg triad, also commonly referred to as the RKR triad, controls the pH sensitivity of ROMK.^{145,146,147,148} Homology modeling provided evidence that the RKR triad plays an important role in Kir channel gating and overall functionality by developing inter- and intra-subunit interactions, however it only influences pH sensitivity indirectly.¹²⁶

2.8.3.1 THE RKR TRIAD

First, the distance between K80 and the two residues R41 and R311 is rather large at approximately 24 Å which makes it impossible for R41 and R311 to have an effect on the ionization state of K80.⁸ Furthermore K80 appears to be solvent-inaccessible since it is embedded in the lipid membrane.⁸ Alternatively, the E318 residue of ROMK's carboxy-terminus, builds a salt bridge, which is a dynamically stable intra-subunit interaction, with R41 located in the amino-terminus of the channel.⁸ Considering the fact that these residues occur in all human Kir channels, suggests an essential structural and functional role.¹²⁶ The second arginine of the Arg-Lys-Arg triad, the C-terminal residue R311, builds a solid inter-subunit ion pair with E302 on the adjacent COOH terminus.⁸

Due to the fact that mutation of R311 (R311W) leads to Bartter syndrome, the residue plays a crucial role in ROMK.¹⁴⁸

A177T is a mutation of A177 located in TM2 that causes Bartter syndrome as well. The mutation R311W leads to a shift in pK_a of ROMK from around 6.5 to 9.2 and the mutation A177T alters ROMK pK_a from 6.5 to 7.8.⁸ Consequently Kir1.1 becomes malfunctional in physiological pH_i range.^{126,148,135} The distance between A177 in the inner transmembrane region TM2 and K80 in the outer transmembrane region TM1 is smaller than 3 Å. Both residues are located in the “helix bundle crossing” region and MD simulations indicate a potential intra-subunit bond between A177 and K80.¹²⁶ It should be noted that mutations in all 6 just mentioned residues (R41, R311, K80, E302, E318, A177) lead to alteration in pH_i sensitivity of ROMK.

2.8.3.2 THE HYDROPHOBIC RESIDUE L160

ROMK possesses four leucines designated L160, which are the equivalent to phenylalanines of KirBac1.1 in the bundle-crossing region.⁸ Therefore it can be assumed that steric blockage of the permeation pathway by the convergence of all four leucines at the cytoplasmic peak of the inner transmembrane helices (TM2) leads to the closing of ROMK by acidification.¹⁴⁹ Sackin et al. discovered that by exchanging the leucines with glycines, pH gating is terminated.¹²⁴ This can be explained by the fact, that glycines lack the hydrophobic side-chain, therefore eliminating the possibility of hydrophobic restriction in this area.

2.8.3.3 RESIDUES IN THE TM2 TRANSMEMBRANE REGION

G148 and G157 in Kir1.1b (G167 and G176 in Kir1.1a) are two significant glycine residues located near the cytoplasmic end of the inner transmembrane helix (TM2).¹²³ Replacing one of the two glycines with alanines results in a rise of pK_a from 6.6 to 7.1 or 7.3.⁸ Even though the mutations do not terminate gating, they substantially modify it, which suggests that both residues play a role in the gating mechanism of Kir1.1b.¹²³ When both glycines combined are mutated to alanines an additive effect occurs that shifts the pK_a by 1.2 pH units to approximately 7.8 for the double mutant:

G157A1+G148A.¹²³ Therefore, it can be assumed that through autonomous mechanisms, the mutations stabilize the closed state in comparison to the open state. Whether the two residues influence pH_i sensitivity in ROMK remains unclear.¹²³

In the absence of extracellular K⁺, intracellular acidification results in irretrievable collapse of the ROMK channel pore.⁸ Even though K80 is not necessary for pH inhibition, the residue is required to link the extracellular K⁺ concentration to pH gating, a fact that delivers explanation to why K80 is highly conserved in almost all Kir1.1 channels.¹²⁶ Furthermore, ROMK's sensitivity to extracellular K⁺ is determined by K80. A strong alkaline shift of pH_i sensitivity can be observed in the Kir1.1-A177T mutant channel.¹²⁶ However, by adding the K80I mutation, a rehabilitation from acid inhibition takes place in the absence of extracellular K⁺.¹²⁶ A functional correlation between external K⁺ gating and internal pH gating in the Kir1.1 family was confirmed through a study on the L160G mutation by Sackin et al.¹⁵⁰ It was shown that inactivation and reactivation by external K⁺ can be inhibited by a mutation of the inner pH gate. The L160G mutation causes a faulted pH gate that prevents pH gating as well as inactivation of ROMK by low external K⁺.¹⁵⁰

2.8.3.4 TM1–TM2 H BONDING

Homology modeling of ROMK demonstrates that the already mentioned residues K80 of the outer transmembrane region TM1 and A177 located on the inner transmembrane region TM2 are connected via H bonding in the bundle-crossing region.^{8,125} K80 has a significant impact on Kir channel pH sensitivity which was determined by measuring the pH sensitivity of various K80 mutations and subsequently plotting the obtained data against the τ_{UPIP_2} for PIP₂ activation.¹²⁵ A definite correlation between the pH sensitivity and PIP₂ activation rate (τ_{UPIP_2}) can be observed. A reduction in pH sensitivity was shown by mutations of K80 to other residues which are expected to destroy the H bonding.⁸ Additionally, via alkalization the mutants promoted the channels rehabilitation from blockage by acidification.⁸ Other K80 mutations exhibit intermediate or even high pH sensitivity, which correlates directly with the amounts of PIP₂ activation and consequently with the ability of K80 and A177 to build the previously indicated TM1-TM2 H bond, meaning that residues which are not able to H bond lead to channels with low pH sensitivity.¹²⁵

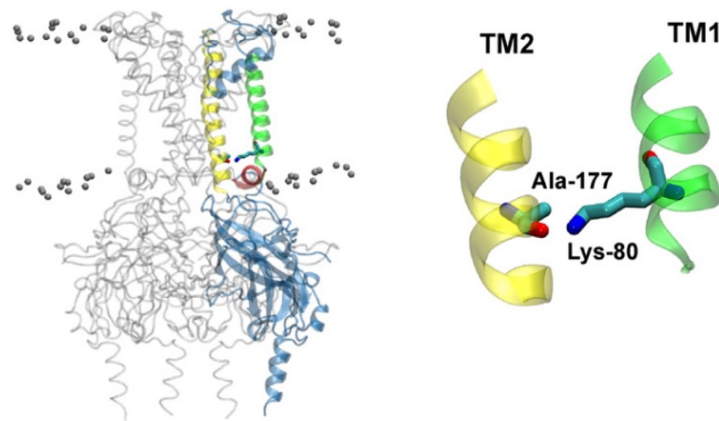


FIGURE 18: TM1-TM2 H BONDING¹²⁵

Side view of the structural ROMK model with one of the four subunits being highlighted. The expanded model on the right visualizes the proximity as well as the orientation of the K80 side yellow chain and the backbone carbonyl of A177. TM2 is displayed in yellow and TM1 is shown in green with the slide helix in red.

2.9 PHARMACOLOGY

2.9.1 NATURAL TOXINS AS ROMK BLOCKERS

Three naturally occurring toxins block ROMK.^{151,152} Tertiapin, the bee venom peptide toxin already mentioned in chapter “2.3: ROMK in the brain” of this thesis, was the first discovered ROMK inhibitor, reaching a 100-fold higher potency for the rat channel than on the human isoform.^{153,154} Tertiapin Q, the synthetic oxidation-resistant derivative of Tertiapin functions as a ROMK channel blocker as well. The honey bee toxin derivative achieves ROMK restriction by binding to the channels external vestibule, an effect dependent on extracellular pH,¹⁵⁵ as the dissociation constant of ROMK’s interaction with Tertiapin Q is decreased in alkaline pH.⁸ Although delta-dendrotoxin (DTX), a mamba snake toxin, is not able to block conduction through the ROMK pore completely, it binds to the channels pore region, thereby inhibiting it.¹⁵⁶ Another ROMK pore blocker is the scorpion venom peptide toxin Lq2, which is an isoform of charybdotoxin (ChTX).¹⁵⁷ The three peptides, which display great affinity ($IC_{50} < 0.5 \mu M$) toward Kir channels, share various similarities with other venom toxins: They possess multiple positively charged residues as well as cysteines.¹⁵² Via docking studies and computational stimulation hypothesis the binding mechanisms of these toxins were established.¹⁵⁸ Electrostatic interactions between ROMK and the animal toxins are enhanced through contact of negatively charged residues on ROMK channel pore region with adjacent positively charged residues from the peptides. Furthermore, binding affinity is supported by hydrophobic bonds among aliphatic amino acids.¹⁵²

2.9.2 SMALL-MOLECULE ROMK INHIBITORS

ROMK’s crucial role in the regulation of kidney functions made the inwardly rectifying potassium channel a popular research topic for major pharmaceutical companies.^{74,153} Since ROMK does both, controlling potassium secretion in the distal nephron as well as mediating sodium reabsorption in the TAL, a new class of diuretics inhibiting ROMK is expected to stimulate diuresis and natriuresis without the unfavorable side effect of potassium wasting, thereby preventing hypokalemia.⁴¹ Especially the Merck Sharp & Dohme (MSD) group is known for its accomplishments in development of ROMK inhibitors as they patented around 20 compounds after exploring multiple hundreds of molecules via high throughput screening.^{153,74}

In 2009, Lewis and colleagues discovered VU590 (7,13-bis(4-nitro- benzyl)-1,4,10-tri-oxa-7,13-diazacyclopentadecane)¹⁵⁹, the first small-molecule with ROMK blocking abilities through screening of approximately 225000 compounds from the NIH Molecular Libraries Screening Center Network (MLSCN) for small-molecule modulators of ROMK by usage of a fluorescence-based thallium flux assay.⁴¹ VU590 blocks the ROMK pore, approaching its binding site through the cytoplasmic channel pore with an IC₅₀ of 240 nM.¹⁵⁹ The functional binding site for VU590 in ROMK, asparagine 171 (N171), which also happens to be the residue responsible for ROMK's weak inward rectification, was established through molecular modeling, patch-clamp analysis and scanning mutagenesis.¹⁶⁰ Additionally, the small-molecule VU590 inhibits Kir7.1 at low micromolar concentrations. Since Kir7.1 and ROMK are coexpressed in the nephron, VU590 was unsuitable as an exclusive ROMK function blocker in the kidney, leading to a search for more selective ROMK inhibitors.¹⁶¹

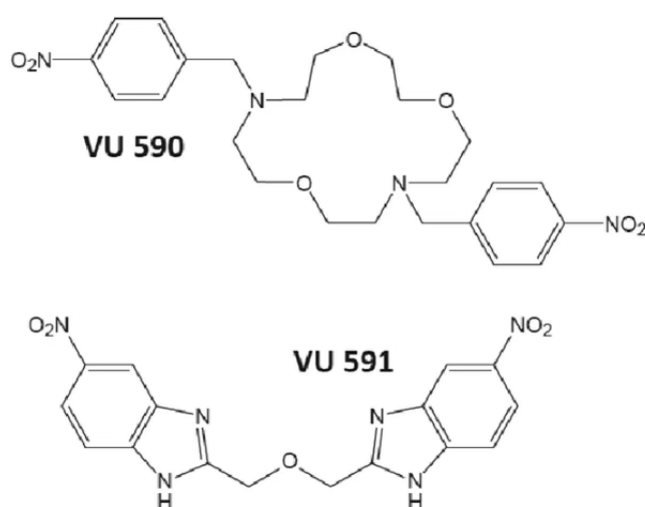


FIGURE 19: CHEMICAL STRUCTURES OF VU590 & VU591⁷⁴

BNBI (bis-nitro-benzimidazole), one of the primary screen hits, shared structural similarities with VU590.⁴¹ Even though the newly discovered compound had no influence on Kir7.1, its ability to block ROMK was weak. After adding an ether oxygen to BNBI, which was one of the main differences between BNBI and the previously mentioned VU590, a 30-fold increase in ROMK blocking potency was achieved without affecting the newly developed compounds selectivity.⁴¹ Like VU590, the new inhibitor VU591 (2,2'-oxybis(methylene)bis(5-nitro-1H-benzo-[d]imidazole)¹⁵⁹ displays potassium- as well as voltage-dependent ROMK block with submicromolar potency by binding to the

channels pore at an intracellular site.^{74,39} The nitro group of VU591 interacts with N171, a hydrogen bond donor.² Meanwhile, the nitro group builds energetically unfavorable interactions with ROMK's negatively charged residues. Explanation for why VU591 selectively inhibits ROMK over Kir7.1 offers the double mutant N171E/K80M, that blocks the inhibitor's activity.¹⁶² Unfortunately, VU591 exhibited unsatisfactory metabolic stability along with high serum protein binding, therefore failing to cause a diuretic effect.⁴¹ Nevertheless, the small-molecule ROMK inhibitor provided a solid starting point for the identification of various effective compounds for use as novel mechanism diuretics.

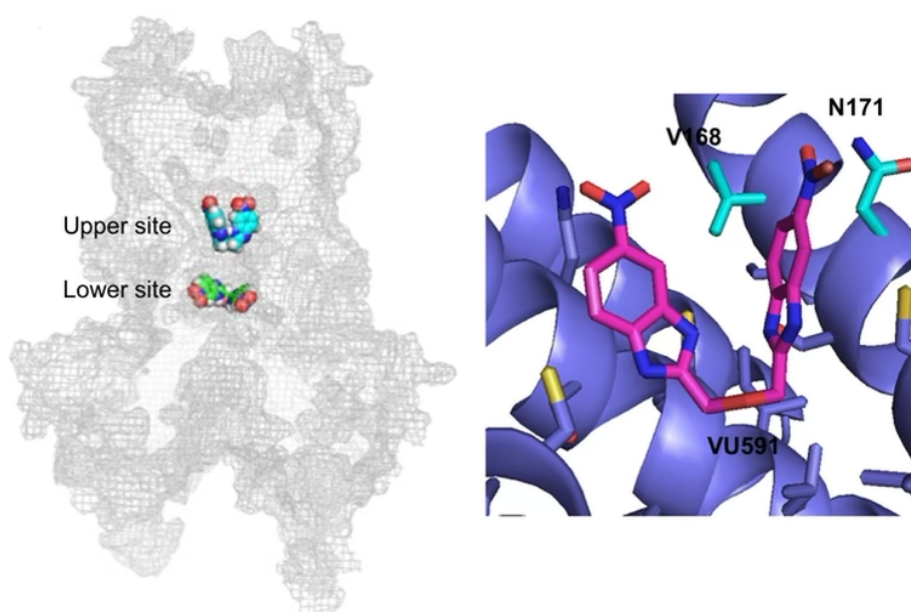


FIGURE 20: IDENTIFICATION OF THE VU591 FUNCTIONAL BINDING SITE IN ROMK⁴¹

To determine energetically beneficial docking sites of VU591, *in silico* docking as well as homology modeling were used. Afterwards, the identified pore locations were verified with patch-clamp electrophysiology and mutagenesis. The image on the right shows a close-up view of VU591 interacting with N171 and V168. The effectiveness of VU591 decreases if any of these two residues is mutated.⁴¹

In 2012, Merck scientists first released their work on the development of those new diuretics varying in structure.¹⁶³ The first effective ROMK inhibitor was called Compound A, a symmetrical small molecule which, as its predecessors VU590 and VU591, needs the residue N171 in order to block ROMK with high affinity.⁴¹ Moreover, Compound A and VU591 both have a nitrogen-containing linker in between two nitro groups. With this new ROMK inhibitor Merck was able to demonstrate that pharmacological ROMK blockage achieves diuresis as well as natriuresis whilst preventing potassium wasting.⁴¹ Furthermore, blood pressure reduction is achieved, which consequently precludes kidney damage by hypertension.¹⁶⁴

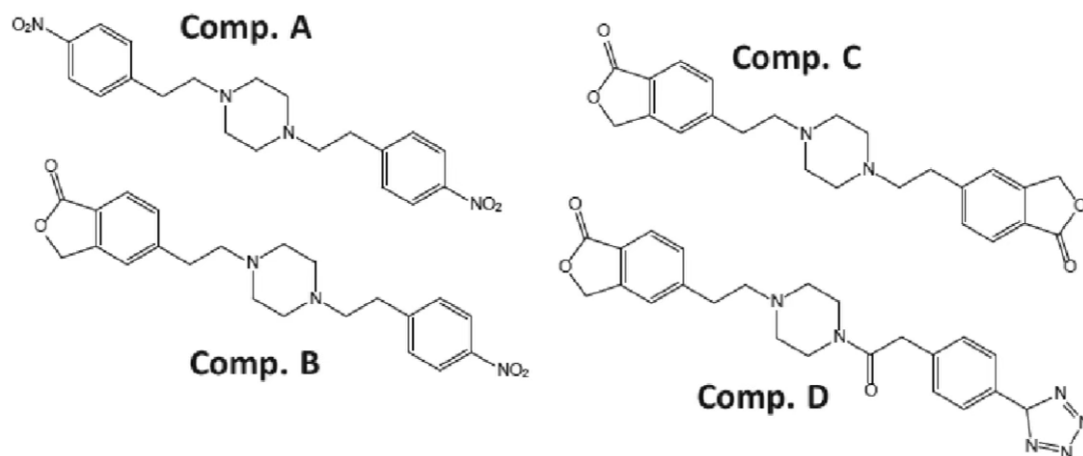


FIGURE 21: CHEMICAL STRUCTURES OF COMPOUNDS A, B, C AND D⁷⁴

Unfortunately Compound A exhibited high potency in blocking the hERG channel, therefore causing a potential risk for cardiac arrhythmia.¹⁶³ In Compound B reduction of affinity for hERG was accomplished by exchanging one of the nitrophenyl groups with a 4-phthalide group.⁷⁴ In Compounds C and D both nitrophenyl groups were replaced. Adding another 4-phthalide group instead of a nitrophenyl group in Compound C resulted in a significant decrease in hERG affinity.⁷⁴ However, the most promising pharmacological feature profile was displayed by Compound D. The piperazine exhibited the least interaction with hERG channels.¹⁵³ Furthermore, diuresis and natriuresis as well as good oral bioavailability was achieved in pre-clinical studies with laboratory animals after short-term administration.¹⁶⁴ Even though Compound D exhibited similar effectiveness as hydrochlorothiazide (HCT), it conveniently did not cause hypokalemia.¹⁶⁴ Interestingly, modifications on the core skeleton of those compounds and alterations in distance between the nitrophenyl groups resulted in a decreased effect on ROMK.¹⁶³

Two of the newest, most promising examples of ROMK inhibitors, based on those above-mentioned pioneer compounds and their structure-activity relationships, are Compound E and MK-7145.¹⁶⁵ The greatest selectivity for ROMK over other potassium channels, especially hERG, was exhibited by Compound E, a pyridazinone containing a spirocyclic center that shares structural similarities with Compound D.⁷⁴ A significant lowering in blood pressure along with an improved preclinical pharmacokinetic profile was achieved by this state of the art ROMK blocker in the spontaneous hypertensive rat model.¹⁶⁶

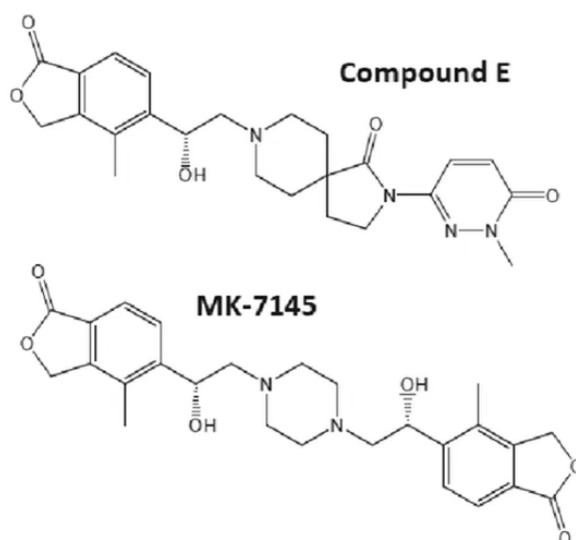


FIGURE 22: CHEMICAL STRUCTURES OF COMPOUND E AND MK-7145⁷⁴

MK-7145, structurally stemming from the di-phthalide derivatives including Compound C, was the first small-molecule ROMK inhibitor clinical candidate.^{165,167} An oral combined administration of MK-7145 with candesartan or hydrochlorothiazide in spontaneously hypertensive rats results in synergistic blood pressure lowering effects.¹⁶⁷ Furthermore, proof-of-concept of MK-7145's therapeutic potential in hypertension as well as congestive heart failure treatment was given after orally administering the ROMK inhibitor to normotensive dogs.¹⁶⁷ Even though no notable shifts in plasma electrolyte levels and in potassium homeostasis occurred, dose-dependent natriuresis along with diuresis was observed. Additionally, after 6 days of treatment an increase in aldosterone and bicarbonate levels was recorded.¹⁶⁷ Suggested by these data, in conclusion pharmacological ROMK inhibition does not only present as a promising new mechanism for treating hypertension and congestive heart failure, but it manifests Bartter syndrome type II features as well.¹⁶⁷

For the treatment of cardiovascular diseases such as hypertension and heart failure but also for other non-cardiovascular disorders and illnesses of the liver and kidney for example, diuretics belong to the most often prescribed classes of drugs.⁷⁴ However, until the discovery of ROMK's favorable pharmacological effects, no breakthrough was accomplished in the field in the last four decades.⁷⁴ Since aldosterone antagonists as well as ENaC inhibitors lead to hyperkalemia, whilst thiazide and loop diuretics cause hypokalemia, ROMK inhibitors exhibit the only diuretics class that does not influence potassium homeostasis significantly.^{74,164} Recent preclinical data shows that ROMK

inhibitors are more effective in lowering blood pressure than thiazide diuretics.¹⁶⁸ Furthermore, through chronic ROMK channel inhibition, an improvement in renal and vascular function as well as recovery from histological harm caused by a high-salt diet, was achieved.¹⁶⁸ Bone mineral density along with plasma lipids were positively impacted. Additionally, salt-sensitive hypertension in humans could soon be a thing of the past, as chronic ROMK inhibition in Dahl salt-sensitive rats reversed hypertension development and end-organ damage.¹⁶⁸

However, before the birth of this newest diuretics class can be celebrated, some obstacles are yet to overcome. Up to now, our understanding of ROMK's pharmacological features is limited. In order to safely establish ROMK inhibitors as a new kind of medication, more data on their interactions with other drugs, their clinical effectiveness as well as the channel's physiology, is required.⁷⁴

3 BARTTER SYNDROME TYPE II

3.1 INTRODUCTION

Bartter's syndrome (BS) is a rare heterogenous group of salt losing tubulopathies with dominant and recessive autosomal inheritance, originally described in 1962 by Frederic Bartter and his colleagues.^{169,170} The kidneys' salt reabsorption disability, meaning they are not able to reabsorb sodium and chloride at the thick ascending limb of Henle's loop, causes exorbitant loss of salt and water from the body along with higher distal salt delivery.¹⁷¹ This leads to extracellular fluid volume reduction, causing activation of the RAAS, the renin-angiotensin-aldosterone system, consequently resulting in secondary hyperaldosteronism. Hyperplasia of the juxtaglomerular apparatus and subsequently hyperreninemia are attributable to permanent stimulation of the RAAS. Bartter's patients usually do not struggle with hypertension, as they maintain low to normal blood-pressure, however elevated prostaglandin E2 (PGE2) levels are associated with the disease. Multiple electrolyte abnormalities characterize the disorder, such as low chloride and potassium and rarely, also hypomagnesemia. Metabolic alkalosis is the acid-base manifestation.¹⁷¹ With a prevalence of 1 in 1 000 000, the disease can be classified as rare.¹⁷²

5 different types of the Bartter syndrome have been classified (Type I-V) based on the genetic defect identified.⁵⁵ For the purpose of this thesis, only Type II Bartter syndrome is of relevance, as it results from homozygous or compound heterozygous loss-of-function mutations in the KCNJ1 gene encoding ROMK. This subtype is commonly described as a severe antenatal form of the disease, usually presenting with polyhydramnios before childbirth.⁵⁵ However, several cases of late-onset presentation have been reported in recent years. To date, there is no cure for Bartter syndrome type II.

3.2 TREATMENT

Medical treatment for Bartter syndrome type II includes potassium supplementation usually administered as oral potassium chloride, aldosterone antagonists like spironolactone, angiotensin-converting-enzyme inhibitors like ramipril and nonsteroidal anti-inflammatory drugs (NSAIDs).⁵⁵ In terms of the effectiveness of these treatments, Nuñez-Gonzalez et al. rated these therapeutic approaches as follows: Supplemental electrolyte drugs are mandatory, principal treatment for Bartter syndrome is indomethacin, potassium sparing diuretics can be used but are not recommended and RAAS axis inhibitors are beneficial especially when a patient suffers from nephrotic damage by NSAIDs.¹⁷³ At this point it should be mentioned that, although this was not the case in any of the case reports of the following analysis, some Bartter patients receive growth hormone (GH) therapy, in order to prevent growth abnormalities. Only if NSAID treatment failed, this therapeutic approach is considered.¹⁷⁴ Moreover, there is poor evidence of efficacy in Bartter syndrome patients.¹⁷³

3.2.1 ORAL SALT SUPPLEMENTATION

In order to return to normal electrolyte levels, water and salt supplementation is usually the first-line approach for Bartter syndrome.¹⁷³ Since both, potassium and chloride levels are usually strongly affected in these cases, the preferred form of salt supplementation is KCl. Oral potassium supplementation normalizes hypokalemia, which often occurs in BS2 patients. Additionally, it releases symptoms of muscle weakness, cramps and fatigue, which was the case in case report number 8 of the analysis down below.¹⁷⁵ In case report number 2 it was shown that treatment with oral potassium chloride betters blood and urine chemistries after a few days. Moreover, weakness and fatigue, serum potassium levels, polyuria and polydipsia were improved over the course of a few weeks.¹⁷⁶

3.2.2 NON-STEROIDAL ANTI-INFLAMMATORY DRUGS (NSAIDs)

NSAIDs inhibit cyclooxygenase (COX) non-selectively. COX plays a crucial role in the synthesis of PGE2, consequently NSAIDs prevent PGE2 elevation, which often occurs in Bartter syndrome patients.¹⁷³ Further beneficial effects include improvement of

hypercalciuria, polyuria, growth retardation, secondary hyperaldosteronism and hypokalemic metabolic alkalosis. For Bartter syndrome treatment indomethacin is the most often prescribed NSAID.¹⁷⁴ Indomethacin plays a crucial role early on in the treatment of this disease and was proven to be very effective in improving nephrocalcinosis through reduction of hypercalciuria. In addition, it's restoring effects on electrolyte abnormalities better disease development.¹⁷³ In case report number 9 of the following analysis, four out of five probands received indomethacin and three patients were under single-drug indomethacin therapy.¹⁷⁷ In this study normalization of PTH levels as well as electrolyte abnormalities was successfully achieved. Furthermore, urine calcium excretion was reduced and improvement of polydipsia, polyuria and growth retardation was achieved by indomethacin.

3.2.3 POTASSIUM-SPARING DIURETICS

The potassium-sparing diuretic most prescribed for Bartter patients is spironolactone. The aldosterone antagonist is typically prescribed in combination with oral potassium supplementation, as was the case in case report number 5 and 7 of the upcoming analysis. The beneficial effects of these diuretics include hypokalemia recovery and blockage of aldosterone, leading to improvement of hyperaldosteronism and secondary hyperkalemia.¹⁷³ The other group of potassium sparing diuretics are Na⁺ channel blockers like amiloride, which exhibited good efficacy in Bartter syndrome patients, even in pregnant women.¹⁷⁸ In view of the fact that these medications are associated with serious risks, like hypovolemia, sparing diuretics are only clinically accepted if other therapeutic approaches lead to no significant improvement.¹⁷³ Besides, potassium-sparing diuretics do not lead to improvement in cases with severe phenotypes.

3.2.4 RENIN-ANGIOTENSIN-ALDOSTERONE SYSTEM INHIBITORS

Hypertrophy of the juxtaglomerular apparatus and hyperreninism in Bartter syndrome are caused by continuous stimulation of the RAAS axis, in an effort to retrieve lost water and salt.¹⁷³ Consequently, a therapeutic approach that inhibits this system made sense. Like sparing diuretics, this type of drugs carry potential risks, such as hypotension and hypovolemia, therefore causing prescription restrictions. ACE inhibitors do not lead to improvement of electrolyte abnormalities. Nevertheless, it has been shown

that these drugs can be more effective than sparing diuretics and can be used in cases of nephrotoxicity with NSAIDs or in patients that are therapy resistant for NSAIDs.^{173,179}

3.3 ANALYSIS OF 22 BS2 PATIENTS

3.3.1 CASE REPORT REFERENCES

1	Isolated nephrocalcinosis due to compound heterozygous mutations in renal outer medullary potassium channel. ¹⁸⁰ Khandelwal et al., 2020
2	A novel compound heterozygous KCNJ1 gene mutation presenting as late-onset Bartter syndrome. ¹⁷⁶ Li et al., 2019
3	Late-onset Bartter syndrome type II. ¹⁸¹ Gollasch et al., 2017
4	A novel compound heterozygous ROMK mutation presenting as late onset Bartter syndrome associated with nephrocalcinosis and elevated 1,25(OH) ₂ vitamin D levels. ¹⁸² Sharma et al., 2011
5	Nephrocalcinosis as adult presentation of Bartter syndrome type II. ¹⁸³ Huang et al., 2014
6	Late-Onset Bartter Syndrome Type II Due to a Novel Compound Heterozygous Mutation in KCNJ1 Gene: A Case Report and Literature Review. ¹⁸⁴ Tian et al., 2022
7	Late-Onset Bartter Syndrome Type II Due to a Homozygous Mutation in KCNJ1 Gene: A Case Report and Literature Review. ⁵⁵ Elfert et al., 2020
8	A novel mutation of KCNJ1 identified in an affected child with nephrolithiasis. ¹⁷⁵ Yang et al., 2022
9	Eight novel KCNJ1 variants and parathyroid hormone overaction or resistance in 5 probands with Bartter syndrome type 2. ¹⁷⁷ Zuo et al., 2020
10	Transient hyponatremia of prematurity caused by mild Bartter syndrome type II: a case report. ¹⁸⁵ Verma et al., 2020
11	Two novel mutations of the gene for Kir 1.1 (ROMK) in neonatal Bartter syndrome. ¹⁸⁶ Vollmer et al., 1997
12	Heterozygous Mutations of The Gene for Kir 1.1 (ROMK) in Antenatal Bartter Syndrome Presenting with Transient Hyperkalemia, Evolving to a Benign Course. ¹⁸⁷ Cho et al., 2003
13	Clinical Case of Pregnancy and Follow-Up of Bartter Syndrome (Type II) with a Novel Mutation. ¹⁸⁸ Vedmedovska et al., 2020
14	Expanding the spectrum of genetic mutations in antenatal Bartter syndrome type II. ¹⁸⁹ Fretzayas et al., 2013
15	Clinical exome sequencing uncovers an unsuspected diagnosis of Bartter syndrome type 2 in a child with incidentally detected nephrocalcinosis. ¹⁹⁰ Saha et al., 2022
16	Identification of compound heterozygous KCNJ1 mutations (encoding ROMK) in a kindred with Bartter's syndrome and a functional analysis of their pathogenicity. ¹⁹¹ Srivastana et al., 2013

3.3.2 CASE REPORT TABLE

case report	age of presentation	sex	DNA sequence change	Amino acid change	genotype	exon	clinical presentation of admission	poly-hydramnios	poly-dipsia	poly-uria	elevated PTH	hypo-kalemia	metabolic alkalosis	growth	hyper-calciuria	nephro-calcinosis	high urine chloride
1	14 y	F	c.146G > A(M) c.657C > G(F)	p.Cys491Yr(M) p.Ser219Arg(F)	heterozygous	2	abdominal pain, nephrocalcinosis	Y	Y	Y	Y	N	N	normal	Y	Y	Y
2	32 y	F	c.212C > T(F) c.701C > T(M)	p.Thr71Me(F) p.Thr234Ile(M)	heterozygous	ND	weakness, polyuria, polydipsia	Y	Y	Y	Y	Y	Y	normal	Y	Y	Y
3	43 y	F	c.197T > A c.875G > A	p.Ile66Asn p.Arg292Gln	heterozygous	2	nephrocalcinosis	ND	Y	Y	Y	Y	N	normal	Y	Y	N
4	8.5 y	F	ND	p.Gly90Trp(M) p.Ile211Ser(F)	heterozygous	ND	enuresis, polyuria, polydipsia	N	Y	Y	N	Y	Y	5th P	Y	Y	Y
5	35 y	M	c.658C > T	p.Leu220Phe	homozygous	ND	lower back pain	ND	N	N	N	Y	Y	normal	Y	Y	N
6	34 y	F	c.619C > A(F) c.922C > T(M)	p.Leu207Ile(F) p.Cys308Arg(M)	heterozygous	5	numbness, hand convulsion	N	Y	Y	N	Y	Y	normal	N	Y	Y
7	26 y	M	c.658C > T	p.Leu220Phe	homozygous	ND	weakness	ND	Y	Y	Y	Y	Y	normal	N	Y	Y
8	6 y	M	c.65G > T(F) c.89G > A(M)	p.Arg22Met(F) p.Cys301Yr(M)	heterozygous	4	cramps, fatigue, muscle weakness	Y	N	N	N	N	N	normal	N	N	N
9	23 y	M	c.865C > T c.1013G > A	p.Gln289* p.Arg338Gln	heterozygous	5	nocturnal enuresis, diabetes insipidus	N	Y	Y	Y	Y	Y	normal	Y	Y	ND
9	5 y	M	c.445G > T c.827G > T	p.Val149Leu p.Ser276Ile	heterozygous	5	nephrocalcinosis, pneumonitis	Y	Y	Y	Y	N	N	normal	Y	Y	ND
9	5 y	F	c.558A > T	p.Lys186Asn	homozygous	5	polydipsia, polyuria, growth retardation	Y	Y	Y	Y	Y	Y	< 1st P	Y	Y	ND
9	2 y	M	c.317T > G c.931C > T	p.Ile106Ser p.Arg311Trp	heterozygous	5	gross hematuria	Y	Y	Y	N	N	N	< 15th P	Y	Y	ND
9	3 months	F	c.206G > C c.268G > C c.634C > T	p.Trp69Ser p.Gly90Arg p.Arg212*	heterozygous	5	severe electrolyte disturbance	Y	Y	Y	Y	N	N	< 10th P	Y	Y	ND
10	birth	F	c.996_999del(F) c.329C > T (M)	p.Glu334Glyfs*35(F) p.Pro110Leu(M)	heterozygous	5	postnatal dehydration, hyponatremia	Y	Y	Y	ND	N	Y	2nd P	Y	N	N
11	birth	F	c.1028C > T(F) c.1219C > T(M)	p.Alal156Val(F) p.Leu220Phe(M)	heterozygous	5	typical neonatal BS2 symptoms	Y	ND	ND	ND	ND	Y	ND	ND	Y	ND
12	birth	F	c.1573C > T(F) c.1631T > C(M)	p.Arg338Stop(F) p.Met357Thr(M)	heterozygous	5	azotemia, hyperkalemia, hyponatremia	Y	ND	ND	ND	Y	Y	ND	Y	Y	ND
13	fetus	M	c.554C>T	p.Pro185Leu	homozygous	ND	premature labour threats, maternal dysproea	Y	N	N	ND	Y	Y	normal	ND	Y	ND
14	birth	M	c.226A > G(M) c.944T > G(F)	p.Lys76Glu(M) p.Val315Gly(F)	heterozygous	5	typical neonatal BS2 symptoms	Y	ND	Y	ND	Y	Y	normal	Y	Y	ND
15	11 y	F	c.658C > T	p.Leu220Phe	homozygous	2	high urine oxalate	N	N	N	ND	N	N	< 3rd P	N	Y	Y
16	4 y	F	ND	p.Ser219Arg p.Leu220Phe	heterozygous	ND	developmental delay, abnormal gait	ND	Y	N	ND	Y	Y	< 3rd P	Y	Y	ND
16	1 y	ND	ND	p.Ser219Arg p.Leu220Phe	heterozygous	ND	urinary tract infection	Y	Y	Y	ND	N	N	ND	Y	Y	ND
16	1 y	ND	ND	p.Ser219Arg p.Leu220Phe	heterozygous	ND	typical neonatal BS2 symptoms	Y	Y	N	ND	Y	Y	ND	Y	Y	ND

Abbreviations: ND: no data, Y: Yes, N: No, P: Percentile, In the "sex" column F stands for "female" and M for "male", whereas in the "DNA sequence change" and the "Amino acid change" column F stand for "father" and M for "mother".

3.3.3 CASE REPORT ANALYSIS

This table displays the data from 16 different case reports of 22 people suffering from Bartter syndrome type II, varying in age, sex and nationality. Two of these case reports, labeled number 9 and number 16, exhibit information from multiple patients, with number 16 even being a family report of three siblings with the disease. In an effort to compare the phenotypes caused by different mutations in the *KCNJ1* gene, it was documented if nine of the most typical Bartter syndrome manifestations, including hypercalciuria, nephrocalcinosis and hypokalemia, occur in each person or not. Furthermore, age, sex, information on the gene mutation and the patients' clinical presentation at admission was recorded. By comparison of the collected data, the following conclusions can be drawn:

Given the fact that out of 22 patients, only 5 exhibited a homozygous genotype, it can be determined that there are more compound heterozygous than homozygous mutations in the *KCNJ1* gene, which makes sense, because the likelihood of having inherited the same versions of a genomic marker from both biological parents is much smaller than inheriting different ones. Although the numbers of male and female patients appear to be quite balanced in this analysis, slightly more women have been documented, which corresponds well with two other Bartter syndrome type II case report series: Out of 19 patients in a phenotype-genotype correlation analysis by Brochard et al. 12 are female¹⁹² and in a case-series description by Finer et al. 9 out of 12 infants with Bartter syndrome type II are female as well.¹⁹³ However, no definite conclusions can be drawn from such a small number of patients and in order to establish if the disease actually presents more often in women or not, more data is needed. Nephrocalcinosis was the Bartter syndrome type II manifestation most often recorded in this analysis, as only 2 out of 22 patients did not present with calcium depositions in the kidney. Approximately half of the individuals exhibit normal growth, while growth retardation was observed in seven patients, who are all under the age of 12. Interestingly, the symptoms most often associated with the disease: polyhydramnios (14/22), polydipsia (15/22), polyuria (14/22), hypokalemia (13/22), metabolic alkalosis (14/22) and hypercalciuria (16/22), were almost all recorded in only around half of the patients, which is probably one of the reasons why the nephropathy is hard to diagnose. Although parathyroid hormone (PTH) levels in 8 out of 22 patients were elevated, there is no documentation of PTH levels in several (9/22) cases. It should be noted though,

that high PTH levels due to persistent hypercalciuria were reported in multiple BS type 2 patients.¹⁹⁴ Similarly to the PTH levels, urine chloride levels were not mentioned in 12 out of 22 cases.

Since exon 5 encodes the majority of ROMK's sequences and it being KCNJ1's most influential putative functional domain,¹⁸⁴ almost all KCNJ1 mutations are located in exon 5, which can also be observed in the case reports of this analysis. The individuals described in these case reports vary strongly in their clinical presentations of admission: Some patients for instance, received an incidental nephrocalcinosis diagnosis, which happened in case reports number 1, 3, 5 and 15 and weakness prior to the diagnosis was documented in three people, case reports number 2, 7 and 8. Atypical symptoms, like lower back pain, numbness and hand convulsion were observed in case report number 5 and 6. Interestingly, only 8 out of 22 patients were diagnosed with the disease before or at approximately one year of age. Several of these case reports were described as late-onset presentations of Bartter syndrome type II, but because patients with the disease are often misdiagnosed and most of the time symptoms present quite early on in people's lives, it can be assumed that at least some of these cases are not actually late-onset ones.

3.3.4 ANALYSIS OF CASE REPORT MUTATIONS

The Leu220Phe mutation was documented in seven patients of this analysis. This variation was reported for the first time by Vollmer et al. in an infant suffering from severe antenatal Bartter syndrome type II in a compound heterozygous state, which is presented in case report number 11.¹⁸⁶ Since the mutation is located close to a PKC phosphorylation site in a regulatory ATP-binding domain, containing a Mg^{2+} -ATP binding motif, it was assumed that the mutation is deleterious.¹⁹⁰ Later the Leu220Phe mutation was found in a homozygous state in an adult with incidentally detected nephrocalcinosis in case report number 5 presenting with a mild phenotype.¹⁸³ Case reports number 7 and 15 report this variant in a homozygous state as well. Although those 3 patients presented with different symptoms at admission, there is one thing they all have in common: All patients with homozygous Leu220Phe mutations from this analysis were diagnosed rather late with Bartter syndrome type II. Whereas the individuals with heterozygous mutations, documented in case reports number 11 and 16, all

presented with symptoms under the age of five. However, this might be a coincidence and to be able to know for sure if there is a correlation, more data from patients with the Leu220Phe variant is required.

Both mutations from case report number 1 are categorized as pathogenic due to the fact that they shift pH gating, thereby inactivating ROMK, as was exhibited in heterologous expression experiments.^{148,180} The compound heterozygous mutations in case report number 4, Gly90Trp and Ile211Ser, can be found in the channel core region and in the ATP regulatory domain.¹⁸² A change from glycine to the hydrophobic tryptophan in the core probably affects tetramer assembly, consequently impacting channel trafficking. Position 211 is in approximation to the cAMP-dependent phosphorylation site S219, therefore a switch from the hydrophobic non-polar isoleucine to the polar serine could influence phosphorylation of ROMK, possibly leading to enhanced channel open probability.¹⁸² Interestingly, ROMK alignment uncovered, that the residues Arg22 and Cys30, which are mutated in case report number 8, are highly conserved among various different species.¹⁷⁵ The amino acid switch from arginine, a basic amino acid, to methionine, a neutral amino acid, reduces the forming of hydrogen bonds. The Arg/Lys/Arg triad conformation could be altered by the Arg22Met variant, causing steric clashes of adjacent residues, consequently leading to structural instabilities. The Cys30Tyr mutation alters a residue side-chain, thereby changing protein structure as well as function.¹⁷⁵

		22	30
		↓	↓
<i>HUMAN</i>	M F K H L R K W V V T R F F G H S R Q R A	R	L V S K D G R C
<i>Danio rerio</i>	M T R S L R E W F K E Y M S E R R G R K N	R	L V T K D G R C
<i>Mus musculus</i>	M F K H L R R W F V T H I F G R S R Q R A	R	L V S K D G R C
<i>Bos taurus</i>	M F K H L R K W F V T R F F G R S R R R A	R	L V S K D G R C
<i>Rattus norvegicus</i>	M F K H L R R W F I I T I F G R S R Q R A	R	L V S K E G R C

FIGURE 23: COMPARISON OF KCNJ1 ENCODED PROTEIN ACROSS SPECIES.¹⁷⁵

In case report number 9 mutations of five different patients are documented. The Arg311Trp mutation of one of these people is located in the Arg/Lys/Arg triad and the shift from a hydrophilic to a hydrophobic residue assumingly causes changes in conformation and pH dependence.¹⁷⁷ Three other mutations from this case report, Gly90Arg, Ile106Ser and Val149Leu can be found in the channel core region, where

variants disrupt pore forming components via defective potassium conductance. Mutations in the amino- and carboxy-terminus of ROMK do not destroy channel function completely. The variant Arg338Gln located near ROMK's carboxy-terminus might be responsible for the mild-phenotype presented by a patient of case report number 9, given that the other variant he carried, Gln289*, is severe. The Lys186Asn mutation can be found close to the channel core region and the transmembrane domain TM2. It is believed to block ROMK's functional expression completely, explaining the severe phenotype of its carrier.¹⁷⁷ Extraordinarily, three variants: Trp69Ser, Gly90Arg and Arg212* were detected in one person. The Gly90 residue is located in ROMK's core region and has been previously reported in case report number 4 as a Gly90Trp mutation.

In case report number 10 a frameshift variant Glu334Glyfs*35 and the missense variant Pro110Leu present in a heterozygous state. Pro110Leu can be found in the extracellular channel loop, a region less conserved than others, consequently mutations in this area are assumed to be better tolerated.¹⁸⁵ Deletion of two base pairs in the frameshift variant located in the carboxy-terminal region is predicted to cause early protein termination 35 amino acids downstream from the deletion. Possibly, the mild presentation of Bartter syndrome in this patient can be explained by the fact that both mutations are located outside of the transmembrane domain.¹⁸⁵

It should be noted, that several case reports point out that the combination of mutations in a heterozygous state is possibly not coincidental, but may affect the tertiary or quaternary structure of proteins, thus determining phenotype severity. This could explain late-onset presentations, as the compound heterozygous state of mutations could have partial intrinsic transport.¹⁸⁴

3.4 STRUCTURAL INTERPRETATION

From the AlphaFold structure prediction server the 3D coordinates of the ROMK channel were downloaded.¹⁹⁵ Overall the structure has a very high confidence score. The mutations can be found all over the ROMK protein, although most of them appear to be located in the cytoplasmic domain. In the 3D model it can be observed, that due to their approximation, Leu220 interacts with Cys308. It should be noted that this model is a monomer, even though the ROMK channel is a tetramer, meaning that therefore interactions with other residues cannot be predicted. Further mutations will induce dynamic changes which can not be interpreted with a static model. In the future, studies including X-ray or CryoEM structures of selected disease mutants are required.

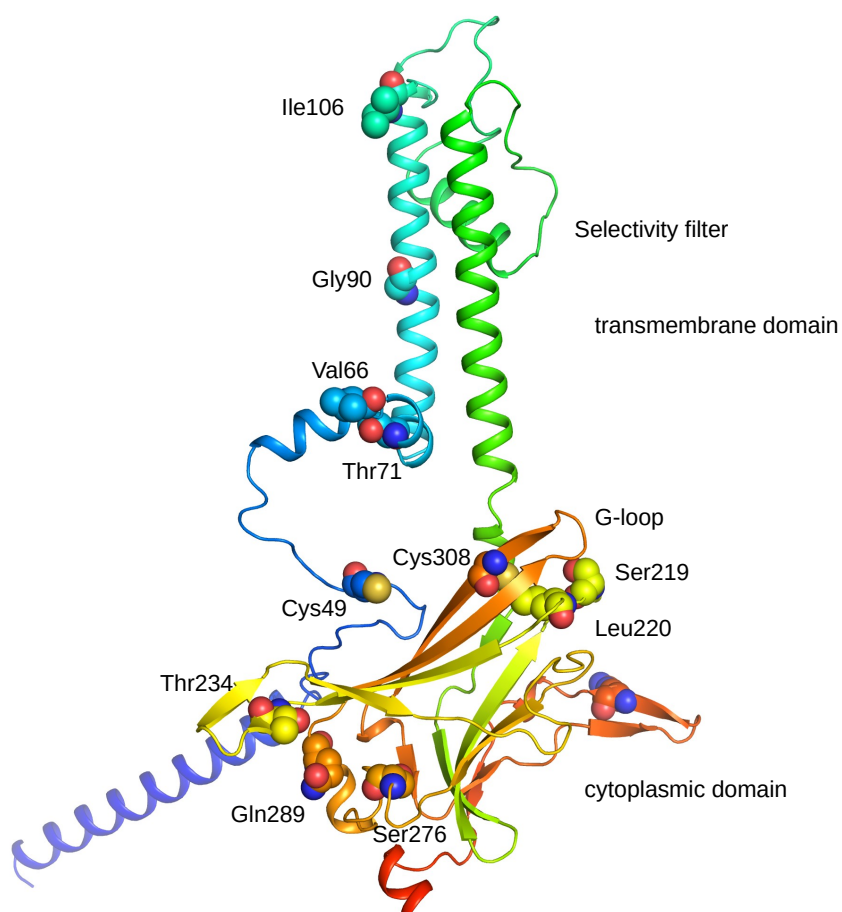


FIGURE 24: ROMK MONOMER^{195,196}

ROMK monomer downloaded from the AlphaFold structure prediction server. The figure was created with PyMOL Molecular Graphic System Version1.8.4.0. Schrodinger LLC.¹⁹⁶

3.5 PREDICTION OF VARIANT PATHOGENICITY

For the most part, the mechanism of ROMK's molecular defects causing Bartter syndrome remain a mystery. In an effort to change that, modern technology tools are used to identify which single amino acid variations impact protein function and further, cause diseases in individuals. Although algorithms for predictive purposes are already available, Ponzoni et al. made it their mission to compare the efficacy of three commonly used and publicly available algorithms: Rhapsody, EVmutation and PolyPhen-2.¹⁹⁷

First, *in silico* saturation mutagenesis of ROMK was performed, meaning assessment of all possible amino acid substitutions at all sequence positions to be able to estimate how they affect protein function. Then the impact of these mutations was examined using Rhapsody, a novel machine learning-based classifier and further prediction tools: EVmutation and PolyPhen-2. Dynamics- dependent properties such as flexibility of residues and further mechanical and allosteric features, additionally to sequence and structure dependent properties are incorporated by Rhapsody.¹⁹⁷ PolyPhen-2 is widely used and was also employed in several case reports of the analysis of this thesis. In a probabilistic classifier the program unites properties deriving from structure with traditional MSA analysis techniques. The coevolution-based algorithm EVmutation evaluates crucial interactions by direct coupling analysis of Pfam domains.

In conclusion it can be stated that deleterious mutations were anticipated successfully by both programs, Rhapsody and EV mutation. Rhapsody prognosticated that more than 90 percent of known Bartter syndrome mutations are deleterious. Interestingly, the two indicated programs display agreement for the most part, even though their methods and criteria vary.¹⁹⁷ Combining forces of programs like these will become more and more valuable as the number of known monogenic diseases increases. Furthermore, evaluations like the previously mentioned one by Ponzoni et al. will aid researchers who want to develop hypothesis on the severity of genetic variants.

4 CONCLUSION

The renal outer medullary potassium channel plays an essential role in the regulation of kidney functions. Its role in the nephron is well established, our understanding of the channel's pharmacological features however, is limited. After four decades of no significant breakthrough in the field of diuretics, ROMK inhibitors could be the first diuretics class without significant influence on potassium homeostasis, promising drastic improvements in the treatment of cardiovascular diseases.

The analysis of 22 Bartter patients suggests a high variance degree of KCNJ1 gene mutations and severity of disease. Up to now it remains unclear how different mutations correlate with the presentation of various phenotypes. Furthermore, this thesis emphasizes, that people affected by Bartter syndrome often get diagnosed after suffering from the disease for many years. Some get misdiagnosed at first and others never receive the right diagnosis at all. In order to find the right therapeutic approach, correct diagnosis is essential. Even though genetic testing leads to accurate differential diagnosis as well as diagnosis of Bartter syndrome type II, the disease remains easily missed and misdiagnosed.

Every published case report is one more step towards understanding ROMK and the disease caused by the channel's mutations. Moreover, with the help of modern technology tools such as Polyphen-2, EV mutation and Rhapsody prediction of variant pathogenicity and consequently treatment of Bartter syndrome could significantly improve.

5 APPENDIX

5.1 LIST OF FIGURES

Figure 1: Kir channel conductance & rectification ⁸	- 1 -
Figure 2: Control of excitability in cardiac cells by Kir channels ⁸	- 2 -
Figure 3: The Kir channel phylogenetic tree ²	- 4 -
Figure 4: Tissue distribution of Kir channels ²	- 5 -
Figure 5: Comparison of subunits: Kir channel vs. K _v channel ⁸	- 6 -
Figure 6: Model of the Kir2.1 channel ³⁵	- 7 -
Figure 7: Schematic illustration of Kir channels' intracellular trafficking routes ³⁵	- 9 -
Figure 8: K ⁺ recycling in the TAL ⁸	- 14 -
Figure 9: Distribution of ROMK isoforms along the nephron ³³	- 17 -
Figure 10: ROMK's regulatory factors ⁷⁴	- 19 -
Figure 11: Regulation of ROMK via SGK1 ⁹³	- 22 -
Figure 12: Important ROMK residues ³³	- 23 -
Figure 13: Atomic ROMK model ³³	- 25 -
Figure 14: The potassium selectivity filter ³³	- 27 -
Figure 15: The transmembrane potassium conduction pathway ³³	- 29 -
Figure 16: The cytoplasmic domains of ROMK ³³	- 30 -
Figure 17: The two G-loop conformations ¹⁰⁷	- 32 -
Figure 18: TM1-TM2 H bonding ¹²⁵	- 38 -
Figure 19: Chemical structures of VU590 & VU591 ⁷⁴	- 40 -
Figure 20: Identification of the VU591 functional binding site in ROMK ⁴¹	- 41 -
Figure 21: Chemical structures of Compounds A, B, C and D ⁷⁴	- 42 -
Figure 22: Chemical structures of Compound E and MK-7145 ⁷⁴	- 43 -
Figure 23: Comparison of KCNJ1 encoded protein across species. ¹⁷⁵	- 53 -
Figure 24: ROMK Monomer	- 55 -

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