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„Lipid interactions and their role in inward rectifier potassium channel regulation“

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1. Acknowledgments

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2. Abstract

Inward rectifier potassium channels (K_{IR}) play important roles in many physiological processes and are essential for maintaining the resting membrane potential. An essential property of K_{IR} channels is that the influx of potassium is more likely than the efflux. Seven subfamilies (K_{IR}1-7) are known to be expressed in different kinds of cells and tissues like the central nervous system, the brain, cardiac cells or skeletal muscles. Mutations cause diseases or syndromes, for example Bartter syndrome, Andersen Tawil syndrome, EAST syndrome, hyperinsulinism or neonatal diabetes. In this master's thesis, we give an overview of different K_{IR} channel families and associated disease-causing mutations. K_{IR} channels are regulated by different kinds of lipids, which occur in the plasma membrane. The main focus of the thesis lies on various lipids and their interactions with different K_{IR} types, especially the role of cholesterol and phosphatidylinositol 4,5-bisphosphate (PIP_2). Cholesterol is known to inhibit the channel activity in most K_{IR} subfamilies. Different putative cholesterol binding sites have been identified and are discussed in comparing them in the different K_{IR} channels. In contrast, PIP_2 acts as an important channel activating factor. Among other phosphatidylinositol phosphates like PI(4)P or $PI(3,4,5)P_3$, $PI(4,5)P_2$ is most likely to lead to an open state of most K_{IR} channels. A primary binding site for PIP_2 has been examined in detail and is part of several crystal structures available. However, the PIP_2 sensitivity depends on the interaction of secondary anionic phospholipids with a secondary binding site. The interplay of PIP_2 and secondary anionic phospholipids with K_{IR} channels results in a conformational shift of the cytoplasmic domain towards the transmembrane domain and the opening of the channel pore. In this thesis, we give an overview of the main PIP_2 binding site and discuss its differences within different available structures. Interestingly, while PIP_2 activates mammalian K_{IR} channels, bacterial K_{IR} channels are inhibited due to differences in key amino acid sequences. To gain insights into this remarkable phenomenon, a structure and sequence alignment were performed to compare the PIP_2 and cholesterol binding sites within different K_{IR} channels.

3. Zusammenfassung

Einwärts gleichrichtende Kaliumkanäle (K_{IR}) haben eine wichtige Funktion für zahlreiche physiologische Prozesse und sind essenziell für die Erhaltung des Ruhemembranpotentials. Eine wichtige Eigenschaft von K_{IR}-Kanälen besteht darin, dass der Einstrom von Kaliumionen gegenüber des Ausstromes favorisiert ist. Sieben Subfamilien (K_{IR}1-7) werden klassifiziert und können in verschiedenen Arten von Zellen und Geweben vorgefunden werden, z.B. im zentralen Nervensystem, Gehirn, Herz oder auch in Skelettmuskeln. Mutationen können zu verschiedenen Krankheiten und Syndromen führen: Beispiele hierfür sind das Bartter Syndrom, das Andersen Tawil Syndrom, das EAST Syndrom, Hyperinsulinismus oder neonataler Diabetes. In dieser Masterarbeit wird ein Überblick über verschiedene K_{IR}-Familien sowie zusammenhängende pathogene Mutationen gegeben. K_{IR}-Kanäle werden über verschiedene Arten von Lipiden, die in der Plasmamembran vorkommen, reguliert. Der Hauptfokus dieser Arbeit liegt in verschiedenen Lipiden und deren Interaktionen mit K_{IR}-Kanälen, besonders aber auf den Aufgaben von Cholesterin und Phosphatidylinositol-4,5-bisphosphat (PIP_2). Cholesterin hemmt die Aktivität von beinahe allen K_{IR} Subfamilien. Es sind bereits verschiedene mögliche Cholesterin-Bindungsstellen identifiziert worden, die hier diskutiert werden in einem Vergleich von verschiedenen K_{IR}-Kanälen. Im Gegensatz dazu zählt PIP_2 als wichtiger Aktivierungsfaktor. Unter anderen Phosphatidylinositolphosphaten wie z.B. PI(4)P oder $PI(3,4,5)P_3$ bewirkt $PI(4,5)P_2$ bei den meisten K_{IR}-Kanälen am ehesten eine Öffnung des Kanals. Eine primäre Bindungsstelle für PIP_2 ist detailliert untersucht und Teil von einigen verfügbaren Kristallstrukturen. Die Sensitivität für PIP_2 hängt von der Interaktion von sekundären anionischen Phospholipiden mit einer sekundären Bindungsstelle ab. Das Zusammenspiel von PIP_2 und sekundären anionischen Phospholipiden mit K_{IR}-Kanälen bewirkt eine Konformationsänderung von der zytoplasmatischen Domäne hin zur Transmembrandomäne, wodurch die Öffnung des Kanals bewirkt wird. In dieser Arbeit wird ein Überblick über die primäre PIP_2-Bindungsstelle gegeben und dessen Unterschiede in verschiedenen verfügbaren Strukturen diskutiert. Während PIP_2 K_{IR}-Kanäle in Eukaryoten aktiviert, werden interessanterweise bakterielle K_{IR}-Kanäle aufgrund

von Unterschieden in wichtigen Aminosäuresequenzen gehemmt. Um Einsicht in dieses bemerkenswerte Phänomen zu bekommen, wurde ein struktureller Vergleich sowie ein Sequenzvergleich durchgeführt, um die PIP_2- und Cholesterinbindungsseite in den verschiedenen K_{IR}-Kanälen zu untersuchen.

4. Inward rectifier potassium channels

Many physiological processes require inward rectifier potassium channels (K_{IR}), which can be found in various kinds of cells, for example in skeletal or cardiac muscle cells as well as in neurons and glia cells. K_{IR} channels have a specific characteristic: the influx of potassium ions can be managed more easily than the efflux. These findings first led to the description as “anomalous” currents of potassium flows because the equation of Nernst anticipated a greater outflow than measured. Today, the process is known as inward rectification. K_{IR} channels play an important role in keeping up the resting membrane potential and differ from usual voltage-gated K^+ (K_v) channels: they do not adhere to Hodgkin-Huxley kinetics.[1] The Hodgkin-Huxley model describes how electrophysical characteristics of membranes depend on the concentration of sodium, potassium and chloride ions.[2] K_{IR} channels are dependent on the electrochemical gradient of potassium ions only. They show a greater flow of ions at a more negative membrane potential. The more it is transformed into positive, the less influx can be measured.[1]

The rectification process is managed by different kinds of blocks on the cytosolic side of the cell, resulting in lower ranges of K^+ efflux. Important molecules and ions for the regulation of the K_{IR} activity are especially polyamines and Mg^{2+}, but also nucleotides, lipids, Na^+ and H^+.[1] As examples for polyamines count spermidine and spermine.[3] Magnesium ions and polyamines can bind to a site located at the intracellular and transmembrane region. The activity of inward rectification differs from type to type and K_{IR} channels can be divided into weak, intermediate and strong types.[1] For example, the $K_{IR}1$, $K_{IR}5$ and $K_{IR}6$ families count as weak types.[4,5] The main reason for these differences is seen in a single amino acid (aspartate), which is located at position 172 in $K_{IR}2.1$.[6] Strong rectifiers (e.g. $K_{IR}2$ and $K_{IR}3$) have aspartic acid at the crucial position for rectifying activity. Weaker rectification activity of $K_{IR}2.1$ has been observed by altering Asp172 to glutamine.[1] The charge of aspartic acid seems to play an important role in the gating mechanism of potassium as well as the blocking process by Mg^{2+}. This has been shown in $K_{IR}1.1$, which has asparagine on the equivalent crucial position.[6] The activity of K_{IR} is also dependent on the concentration of potassium ions on the extracellular side: higher concentrations lead to an increased conductance (except $K_{IR}7.1$). Furthermore, pH

changes can also alter the activity of inward rectification. As intermediate rectifiers count members of the $K_{IR}4$ family.[1]

For some kinds of K_{IR} channels phosphorylation plays an important role. For example, the phosphorylation of Ser44 in Kir1.1 causes a higher expression of channels on the surface of the cell. In this case the phosphorylation is managed by PKA, SGK-1 (serum-glucocorticoid-regulated kinase 1) and aldosterone-induced kinase.[7]

Another important effect on the function of K_{IR} shows phosphatidylinositol 4,5-bisphosphate (PIP_2), which will be described in detail later (see chapter 7).[1] A visualisation of a K_{IR} channel in general with potassium ions flowing through is presented in Figure 1.[4]

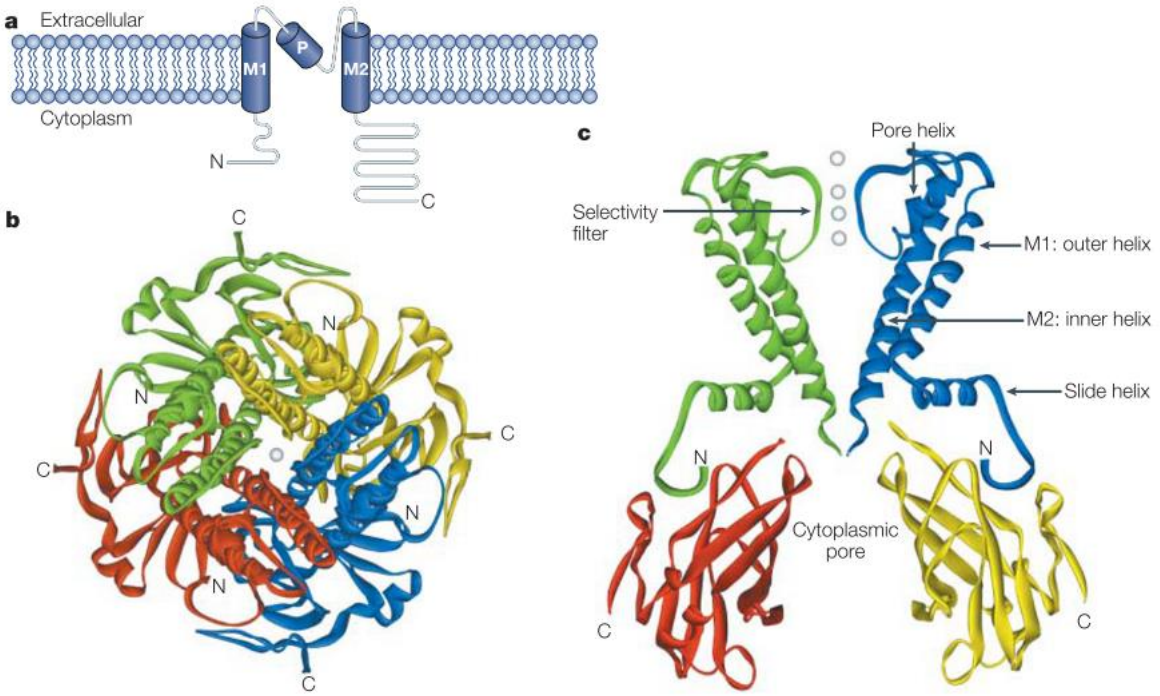


Figure 1: The organisation of K_{IR} channels in general and their composition can be seen in a. B shows a K_{IR} channel with its four subunits and potassium ions in the middle from the top view. C depicts a K_{IR} channel from the side view with potassium ions passing the selectivity filter. Taken from Bichet et al., 2003.[4]

4.1. K_{IR} channel families and their expression systems

Inward rectifier potassium channels can be divided into seven subfamilies. Four functional groups can be found among them. All types consist of a basic structure: two transmembrane domains called *TM1* and *TM2,* which are linked by a region on the extracellular side (*H5*). This part forms a pore and acts as a filter for the ion selectivity, while TM2 faces the inside of the pore. The carboxy- and amino-terminal regions are both located on the inside of the cell.[1,8] The amino acid sequence for this filter activity is well preserved and comes with almost all types of potassium channels like voltage gated potassium channels (K_V): TXGFG or TXGYG. The selectivity for potassium is based on this region because of its narrowness.[4] K_{IR} channels show more than one binding site for polyamines and magnesium ions as blocking agents. An example for an important amino acid is serine in $K_{IR}2.1$, located on TM2 at position 165. It is essential for the binding of Mg^{2+}. All inward rectifier potassium channels build tetramers out of the described subunits.[1,8]

4.1.1. $K_{IR}1.1$

$K_{IR}1.1$ can be found in brain tissue, but primarily in the kidney. It shows weak rectifying activity and is also called renal outer medullary potassium channel (ROMK).[8] $K_{IR}1.1$ counts as an ATP-sensitive potassium channel.[1] An important function is its involvement in water and salt homeostasis together with the Na^+-K^+-$2Cl^-$-cotransporter.[9]

4.1.2. $K_{IR}2.x$

The $K_{IR}2$ family consists of the following types of channels: $K_{IR}2.1$, $K_{IR}2.2$, $K_{IR}2.3$, $K_{IR}2.4$ and $K_{IR}2.6$.[8,10,11] They have strong rectifying activity and are expressed in the nervous system as well as in cardiac cells.[8] $K_{IR}2.4$ can be particularly found in the retina. Its expression on the surface as well as basal current are dependent on heterotrimeric G-proteins. Both is increased by Gβγ and reduced by Gα, which is sensitive to pertussis toxin.[10] $K_{IR}2.x$

channels are characterized by being constitutively active and by having a quite negative resting membrane potential (E_{res}). At first, they were thought to build only homotetramers but heterotetrameric complexes have already been shown as well. The way of K_{IR}2.x channels to find their right position onto the surface of the cell from the endoplasmic reticulum (ER) via Golgi apparatus is defined by a certain amino acid sequence, which differs to the one from K_{IR}1.1. This finding presumes that there exists a different signalling for every single family. K_{IR}2.x channels are classical K_{IR} channels.[1] K_{IR}2.6 is a rather new member of the K_{IR}2 family and can be found in skeletal muscle. Its expression is regulated by thyroid hormone; the transcription of the corresponding gene KCNJ18 is increased by triiodothyronine.[12] Mutations can cause unphysiological excitability and anomalous current signatures and are associated with thyrotoxic periodic paralysis. Despite sharing amino acid sequences for endoplasmic reticulum- and Golgi apparatus signalling with K_{IR}2.1 and K_{IR}2.2, K_{IR}2.6 is mostly held back in the ER. It can build inward rectifier channels with other subunits like K_{IR}2.1 and K_{IR}2.2. In co-assembly with other subunits, K_{IR}2.6 is responsible for less channel expression on the cell surface. So, it primarily works as a regulator for K_{IR}2.x channels in general. The K_{IR}2.6 genes match 98,6 identity with K_{IR}2.2, which has been proved in a sequence alignment.[11]

4.1.3. K_{IR}3.x

Another family with strong rectifying activity represents the K_{IR}3 family, which counts four different subtypes (K_{IR}3.1-K_{IR}3.4). All types are regulated by G-proteins. Gβγ can activate the channels by binding to the C- or the N-terminus of the channel. K_{IR}3.x channels are expressed in cardiac cells, pancreas and the nervous system in general.[8] The channels of this K_{IR} family are also called GIRK. They can occur as homo- or heterotetrameric channels, where combinations of segments from various GIRK channels can be found. GIRK1 is different from the others because of its long C-terminal tail. A channel composed of GIRK2 and GIRK3 has been analysed in a study by Jelacic et al. and is assumed to occur in the brain.[13] There are different types of receptors which can lead to an activation of this G-protein gated process, for example $GABA_B$, 5-HT_{1A}, D_2, P_1 or M_2. Heterotetrameric channels with GIRK1 as a component show a 5-fold higher sensitivity to being activated by

Gβγ than GIRK2/GIRK3 channels.[13] In absence of any activating ligand, $K_{IR}3$ channels remain closed. The responsible amino acid for this mechanism in $K_{IR}3.4$ is a glycine located at position 175. If it is experimentally replaced with proline, the channels remain opened permanently.[1]

4.1.4. $K_{IR}4.x$ and $K_{IR}5.1$

Heterotetramers can generally be found among representatives of one subfamily, e.g. GIRK2/GIRK3. An exception represents a channel consisting of subunits from $K_{IR}4.1$ and $K_{IR}5.1$. The composition of K_{IR} channels is important for their function and location in the cell. Inward rectifier potassium channels in general can occur on apical or basolateral sides as well as pre- or postsynaptic.[1] $K_{IR}4$ and $K_{IR}5$ are expressed in the brain. $K_{IR}4$ can occur as a homomeric channel or can be combined with $K_{IR}5$ to form a 4-4-5-5 or a 4-5-4-5 channel.[8]

4.1.5. $K_{IR}6.1$ and $K_{IR}6.2$

The $K_{IR}6$ family consists of three types: $K_{IR}6.1$, $K_{IR}6.2$ and $K_{IR}6.3$. They are described as ATP-sensitive potassium channels (K_{ATP}). While $K_{IR}6.1$ and $K_{IR}6.2$ occur in mammalians, $K_{IR}6.3$ has been discovered only in zebrafish.[14] K_{ATP} channels are heteromultimers and consist of $K_{IR}6.x$ subunits to form a channel pore together with sulfonylurea receptors (SUR), which are responsible for the regulation and activity. Two different genes of SUR have been described. The most important appearance of these channels seems to be in the pancreatic β-cells as they regulate insulin secretion, but they are also expressed in skeletal, smooth muscular, cardiac cells and neurons. Open K_{ATP} channels are responsible for setting the resting membrane potential. The closing of the channels leads to a depolarization of the plasma membrane caused by other channels. $K_{IR}6$ channels count as weak rectifiers but homologue to the example of the mutation of $K_{IR}2.1$ at position 172, $K_{IR}6.2$ can experimentally be turned into strong rectifiers by changing asparagine at position 160, which is located on TM2, to aspartic acid.[15]

4.1.6. $K_{IR}7.1$

$K_{IR}7.1$ shows some differences to other inward rectifier potassium channels. Other channels than $K_{IR}7.1$ have an arginine in the pore region but this type displays methionine instead at position 125. This results in a different property: the single-channel conductance decreases. The positive charge of Arg operates as a barrier for cationic blockers coming from the outside of the cell. Furthermore, $K_{IR}7.1$ is expressed in exceptional tissues like the intestine (more precisely in epithelial cells) and the thyroid gland (in follicular cells). These findings suggest that the described channel has an important function coupled with Na^+-K^+-ATPase. Regarding the presence of $K_{IR}7.1$ in the thyroid gland, it may be possible that the channel contributes indirectly to the transport of ions like I^-.[3] Another study by Papanikolaou et al. has found that $K_{IR}7.1$ is also expressed in oligodendrocytes (regarding the optic nerve of mice); the frequency of transcript levels is similar to the ones of $K_{IR}4.1$.[16]

4.2. Sequence alignment

K_{IR} type	**gene**	**UniProtKB**	**K_{IR} type**	**gene**	**UniProtKB**
$K_{IR}1.1$	KCNJ1	P48048	$K_{IR}3.3$	KCNJ9	Q92806
$K_{IR}2.1$	KCNJ2	P63252	$K_{IR}3.4$	KCNJ5	P48544
$K_{IR}2.2$	KCNJ12	Q14500	$K_{IR}4.1$	KCNJ10	P78508
$K_{IR}2.3$	KCNJ4	P48050	$K_{IR}4.2$	KCNJ15	Q99712
$K_{IR}2.4$	KCNJ14	Q9UNX9	$K_{IR}5.1$	KCNJ16	Q9NPI9
$K_{IR}2.6$	KCNJ18	B7U540	$K_{IR}6.1$	KCNJ8	Q15842
$K_{IR}3.1$	KCNJ3	P48549	$K_{IR}6.2$	KCNJ11	Q14654
$K_{IR}3.2$	KCNJ6	P48051	$K_{IR}7.1$	KCNJ13	O60928

Table 1: Analysed K_{IR} types with their corresponding gene and UniProtKB.

A sequence alignment has been performed using the Clustal Omega programme via uniprot. The analysed K_{IR} channels with their corresponding gene are presented in Table 1. The result of the alignment is shown in the appendix. The calculation shows an identity of 6.8% and finds 39 identical positions as well as at least 83 similar positions. It is

noticeable that the site, which is responsible for the pore block by magnesium ions and the gating mechanism via polyamines, is placed at the same functional position of each channel. Each site consists of only one amino acid. The site for $K_{IR}2.4$ and $K_{IR}2.6$ is not defined on uniprot but as they have aspartic acid on the same position as the other members of the $K_{IR}2$ family it is likely that D177 ($K_{IR}2.4$) and D173 ($K_{IR}2.6$) are the active sites. This provides evidence that channels, which count as strong rectifiers like $K_{IR}2$ members, have aspartic acid on this crucial position: D172 ($K_{IR}2.1$), D173 ($K_{IR}2.2$), D164 ($K_{IR}2.3$), D177 ($K_{IR}2.4$), D173 ($K_{IR}2.6$). Members of the $K_{IR}4$ family, known as intermediate rectifiers, express glutamic acid at positions 158 ($K_{IR}4.1$) and 157 ($K_{IR}4.2$). $K_{IR}7.1$ expresses glutamic acid as well at position 149 ($K_{IR}7.1$) but has weak inwardly rectifying activity.[17] ROMK, a weak rectifier, has asparagine at position 171.[4] This does not seem quite meaningful because of the strong rectifying channels of the $K_{IR}3$ family, which express asparagine at the site as well: N182 ($K_{IR}3.2$), N150 ($K_{IR}3.3$) and N179 ($K_{IR}3.4$). $K_{IR}6.1$ (at position 170), $K_{IR}6.2$ (160) and $K_{IR}5.1$ (161) also have asparagine at the positions described in the brackets.

The long carboxy terminal tail of $K_{IR}3.1$ is clearly noticeable in the sequence alignment. $K_{IR}3.1$ has the longest sequence of all channels as it consists of 501 amino acids. What has been described before can be seen in the sequence alignment as well: the well-preserved motif for the filter region is expressed in all K_{IR} channels with only small differences.[4] The sequence consists of five amino acids. $K_{IR}2.4$ shows a small difference to the others: the first amino acid of the sequence is serine instead of threonine (S147). It could be presumed that this difference does not have a big negative impact on the function of $K_{IR}2.4$ in comparison to other channels because both amino acids do not differ much in their polarity. The hydroxyl group of both serine and threonine is important for their properties and they differ in only one methyl group. The following amino acid of the sequence in every channel (except for $K_{IR}6.1$ and $K_{IR}6.2$) are isoleucine, glycine, tyrosine and glycine. The two mentioned channels have phenylalanine instead of tyrosine. Again, the difference of phenylalanine and tyrosine is relatively small as both are aromatic amino acids. Tyrosine has one hydroxy group in para position in comparison to phenylalanine, which makes tyrosine less hydrophobic.

The sequence alignment enables depicting the relations among K_{IR} channels based on their similarity. A diagram is presented on Table 2. It is noticeable that each family shows a common origin. For example, it can be seen that $K_{IR}2.6$ shares more amino acid sequences with other members of the $K_{IR}2$ family than with e.g. $K_{IR}4.2$. An alignment of $K_{IR}6.1$ and $K_{IR}6.2$ shows an identity of 63.8%, the $K_{IR}2$ family shares 40.7% identity, GIRK channels are only 35.0% ident, wheras $K_{IR}4.1$ and $K_{IR}4.2$ share 59.4% of the sequences.

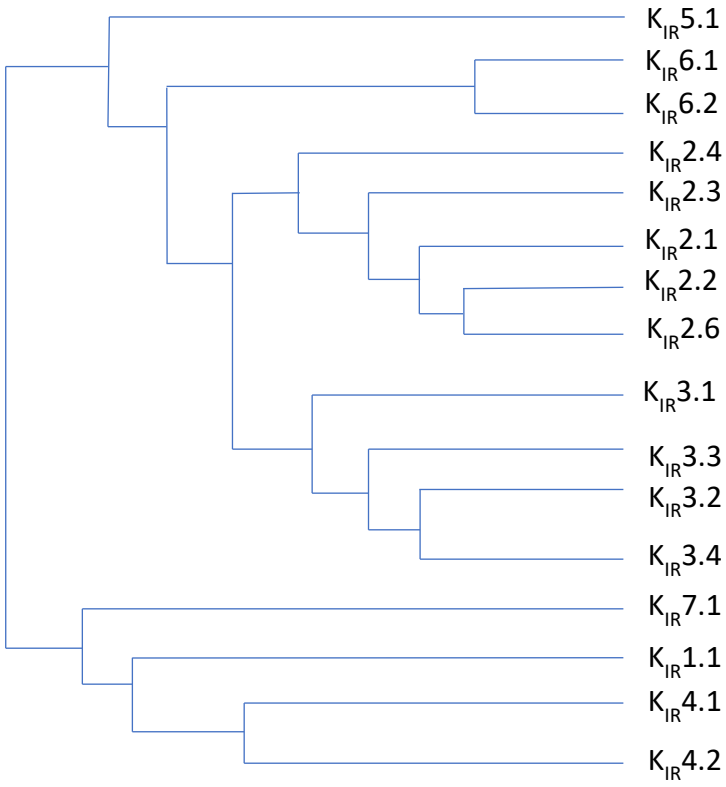


Table 2: A diagram depicting the similarity of K_{IR} channels.

4.3. Physiology and associated diseases

Mutations in K_{IR} channels are often the source of different medical conditions. An overview of selected described diseases is given in Table 3.

Affected channel	Corresponding gene	Disease/syndrome
$K_{IR}1.1$	KCNJ1	Bartter syndrome
$K_{IR}2.1$	KCNJ2	Andersen Tawil syndrome
$K_{IR}2.6$	KCNJ18	Thyrotoxic periodic paralysis
$K_{IR}3.2$	KCNJ6	Keppen-Lubinsky syndrome
$K_{IR}4.1$	KCNJ10	EAST syndrome
$K_{IR}6.1$	KCNJ8	Cantú syndrome
$K_{IR}6.2$	KCNJ11	Hyperinsulinism Neonatal diabetes
$K_{IR}7.1$	KCNJ13	Snowflake vitreoretinal degeneration Leber congenital amaurosis

Table 3: Diseases and syndromes caused by mutations in inward rectifier potassium channels.

4.3.1. Bartter syndrome

The *Bartter syndrome* is caused by a dysfunctional $K_{IR}1.1$ channel. ROMK is responsible for the excretion of potassium in the kidneys. The channel is located on the apical side and manages the potassium efflux in order to keep the Na^+-K^+-$2Cl^-$-transport working. $K_{IR}1.1$ shows different isoforms called ROMK1, ROMK2 and ROMK3, which are different in their amino acid sequences forming the amino terminus of the channel.[9] A sequence alignment of the isoforms shows 95.1% identity. These forms are generated by alternative splicing and have different functions at distant sites along the nephron. Basically, they play an important role for salt homeostasis. ROMK is regulated by an interaction of ATP binding, PIP_2 and phosphorylation by PKA as well as pH on the intracellular side. If ROMK does not work correctly, which may have many different genetic origins, patients suffering from Bartter syndrome experience symptoms like polyuria, hypotension, hypokalaemia and

even metabolic alkalosis. The reabsorption of salt is severely reduced. Type II of this disease is related to a loss of function in ROMK. Hypokalaemia at first seems controversial, as $K_{IR}1.1$ manages the excretion, but can be explained easily. It has been discovered that ROMK knock out mice produced extensively more big potassium (BK) channels, which excrete potassium in such a high amount that hypokalaemia occurs. To be mentioned, hypokalaemia is not present at the beginning, as it can be seen in new-borns suffering from this disease. At first, they show hyperkalaemia, which later leads to transient normal potassium levels until they develop hypokalaemia. Bartter syndrome is inherited autosomal recessively.[9]

4.3.2. Andersen-Tawil syndrome

A loss of function in $K_{IR}2.1$ results in the quite rare *Andersen-Tawil syndrome*. Patients suffer from ventricular arrhythmia, recurrent paralysis and may show physical modifications like hypertelorism, a smaller mandible, a shorter stature or low-set ears. Not every mutation in the KCNJ2 gene displays the same clinical features. The skeletal muscle phenotype does not show dysfunctions in the heart and vice versa does the cardiac type. Arrhythmias, abnormalities in QT- and QU as well as anomalous U waves have been detected in an ECG in a study by Kokubun et al.[18] They examined two meaningful mutations (R67W and R218Q), although more than 40 different genetic modifications are known.[18]

4.3.3. Thyrotoxic periodic paralysis

Mutations in $K_{IR}2.6$ are often associated with *thyrotoxic periodic paralysis (TPP)*. This possibly lethal medical condition comes together with hyperthyroidism and causes attacks that can range from moderate weakness to an entire paralysis. Most patients recover from their symptoms within three days. Hypokalaemia always occurs due to an increased activity of Na^+-K^+-ATPase as well as a reduced efflux of potassium ions. In contrast, more K^+ ions can be found intracellular. TPP often occurs during the night-time

and is associated with triggering factors like exercise, alcohol or heavy meals. In many cases it is unknown that the patient suffers from hyperthyroidism in general as typical hyperthyroid symptoms do not necessarily occur. Normally, TPP does not appear in euthyroid persons.[19]

4.3.4. Keppen-Lubinsky syndrome

Keppen-Lubinsky syndrome occurs when GIRK2 channels do not work correctly by e.g. a loss of T152 caused by a deletion of three nucleotides in KCNJ6. This rare channelopathy causes severe symptoms: patients are intellectually disabled, have a delay in their development, suffer from lipodystrophy, hypertonia as well as hyperreflexia and show different physical anomalies like an open mouth, a high palate, microcephaly and large prominent eyes. Many patients have seizures since their early years. One observed mutation (G156S) is located in the selectivity filter region. This mutation leads to an altered selectivity for potassium ions, which plays an important role for the correct function of K_{IR} channels.[20]

4.3.5. EAST-syndrome

Mutations in KCNJ10, which encodes for $K_{IR}4.1$, can result in the *EAST-syndrome*, an autosomal recessive inherited disease. It is marked by ataxia, tubulopathy, epilepsy and sensorineural deafness. A study by Bockenhauer et al. revealed two specific mutations: G77R and R65P.[21] The patients in this study were normotonic but suffered from a renal salt-loss and a hypokalaemia with metabolic alkalosis. Epilepsy had occurred in infancy for the first time and ataxia was severe. The localisation of these symptoms can be explained because of the expression of $K_{IR}4.1$ in the inner ear, kidney and brain. The evaluated missense mutations show decreased currents of potassium ions.[21]

4.3.6. Cantú syndrome

Cantú syndrome is caused by mutations in the KCNJ8 gene, encoding for $K_{IR}6.1$. As this channel is related to SUR subunits, mutations in the corresponding gene also lead to this disease. Some characteristics of Cantú syndrome like hypertrichosis do not need clinical interventions. Most patients suffer from an enlarged heart with a normal function and half of them have a patent ductus arteriosus, which may need surgical intervention. Symptoms like pulmonary hypertension, skeletal and vascular abnormalities, oedema, acromegaloid facial appearance, behavioural problems and intellectual deficiency may occur. The number of patients is not known exactly but the disease is a rather rare condition. Reported mutations in $K_{IR}6.1$ are V65M and C176S.[22]

4.3.7. Hyperinsulinism and neonatal diabetes

Other important medical conditions concerning the $K_{IR}6$ family are *hyperinsulinism* and *neonatal diabetes* caused by dysfunctional $K_{IR}6.2$ channels or SUR1 subunits. K_{ATP} channels play an important role in secreting insulin from pancreatic β-cells. A study by Shimomura et al. reported mutations in the gating loop.[23] For example, T294M results in hyperinsulinism caused by a decrease of currents. Vice versa, an increase leads to neonatal diabetes combined with a delay of the patients' development. One possible mutation for this condition is T293N. The closure of K_{ATP} channels managed by ATP is important for the depolarisation and therefore the Ca^{2+} influx, which causes insulin secretion. If $K_{IR}6.2$ channels are opened, the plasma membrane hyperpolarizes, and insulin cannot be released. Hyperinsulinism can lead to dangerously low plasma glucose levels, causing brain damage. Common medical conditions related with this type of hyperinsulinism are muscle weakness and epilepsy.[23] Namely, the DEND (developmental delay, epilepsy, neonatal diabetes) syndrome can occur within hyperactive $K_{IR}6.2$ channels.[24]

4.3.8. Snowflake vitreoretinal degeneration and Leber congenital amaurosis

Two different retinal disorders, snowflake vitreoretinal degeneration and Leber congenital amaurosis (LCA) can occur as a result of a dysfunctional TM2 domain in $K_{IR}7.1$. The related gene is KCNJ13. The homotetrameric channel is especially expressed in the retinal pigment epithelium. $K_{IR}7.1$ is assumed to play an important role in the recycling process of potassium ions as it occurs together with the Na^+-K^+-pump. Patients suffering from Leber congenital amaurosis are blind due to a loss of photoreceptors and retinal dystrophy. In addition, they have amaurotic pupils, nystagmus and on an electroretinogram they do not show electrical signals. Snowflake vitreoretinal degeneration is less severe than LCA: the retinal function is only moderately anomalous, and patients have a normal visual acuity. Cataracts can occur early and the disease is characterized by small deposits on the retina, reminding of snowflakes.[25]

4.3.9. Mutations concerning the PIP_2 interaction

Several mutations in $K_{IR}2.1$ leading to Andersen-Tawil syndrome have been identified and some of them affect the K_{IR}-PIP_2 interactions, for example R218W or R218Q. A change of charged to uncharged residues results in a loss of function, as it has been observed in *in vitro* experiments by Lopes et al.[26]

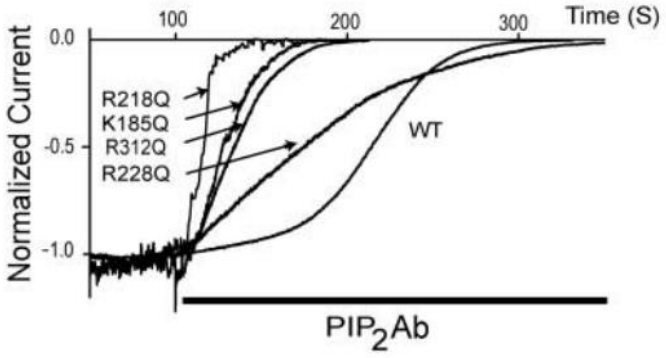


Figure 1: A comparison of the normalized current of wild type $K_{IR}2.1$ and several mutations via inside-out patches after applying PIP_2 antibodies (PIP_2Ab). The currents were studied at -80mV. Taken from Lopes et al., 2002.[45]

Andersen-Tawil syndrome can occur sporadically or may have an autosomal dominant origin, where wild type K_{IR} channels are often expressed together with the mutant channel. Figure 1 shows the results of an *in vitro* experiment, where the effects of four different mutations have been investigated: basic amino acid residues (R218, K185, R312 and R228) were altered to glutamine (uncharged) and PIP_2 antibodies were added. As PIP_2

is an important activating factor in K_{IR} channels, PIP_2 antibodies lead to an inhibition. As the wild type has the strongest interactions with PIP_2, its current inhibition was slower than in the mutated channels. To ensure an acceptable comparability, the time, in which the current is decreased to its half (T_{50}, -50 in the Figure) has been measured. Another *in vitro* experiment of wild type $K_{IR}2.1$ and the R218Q mutation expressed in Xenopus laevis oocytes showed, that the wild type channel was activated almost to its maximum by adding 25µM PIP_2, while the activation of the mutant was noticeably lower. The EC_{50} of the wild type was observed to be approximately 4.6µM (of PIP_2), 12µM for a 1:1 mutant and wild type co-expression and 19µM for a 4:1 ratio. Unitary currents have been examined as well. Expressions of R218Q alone showed no channel activity at all, co-expressions with the wild type led to changes in the channel gating properties.[26]

A similar mechanism has been seen in $K_{IR}1.1$, when R311 (which is equivalent to R312 in $K_{IR}2.1$) is altered to tryptophan or glutamine. These mutations are known to cause forms of Bartter syndrome. The equivalent of R218 in $K_{IR}2.1$ is R217 in $K_{IR}1.1$.[26]

Other mutations concerning the K_{IR}-PIP_2 interactions are V59G and V59A in $K_{IR}6.2$. The residue is located in the slide helix, the equivalent in $K_{IR}6.1$ is I60. The V59G mutation causes fully active channels. PIP_2 is unable to activate the channel even more and therefore results in an insensitivity to PIP_2. In contrast, V59A mutated channels could be activated a bit by PIP_2.[24] The R176C mutation in $K_{IR}6.2$ causes a reduction in the PIP_2 binding and has been associated with type 1 diabetes in a case study by Edghill et al.[27] The interaction of PIP_2 with $K_{IR}6.2$ plays an important role in the insulin secretion. Interestingly, some family members of the patient showing the same mutations did not suffer from diabetes.[27]

Concerning $K_{IR}7.1$, a mutation (R162W) has been found to decrease PIP_2 binding and leading to Snowflake vitreoretinal degeneration. It is presumed that the tethering of the cytoplasmic domain towards the transmembrane domain is prevented by the change of the charged lysine to the uncharged tryptophan, which leads to a loss of function.[17]

5. Lipids

5.1. Classification

Lipids are generally known as lipophilic or partial amphiphilic molecules. A classification system counts eight different kinds of lipids. All the defined molecules occur in prokaryotic as well as eukaryotic cells. Lipids can be divided into two major groups: the ones that are built up of isoprene units like prenol and sterol lipids and the ones that consist of condensations of thioesters, based on carbanions. The second group counts fatty acyls, polyketides, sphingolipids, glycero(phospho)lipids and saccharolipids. It is estimated that the diversity of lipids counts over tens of thousands of different structures. The described categories are divided into at least one main group and one subgroup. If necessary, additional groups can be found in the classification of specific lipids.[28] The way lipids are built up of hydrophobic and often also hydrophilic parts results in their specific classification.[29]

Fatty acyls show a relatable elementary structure, as they are built up of malonyl-CoA and acetyl-CoA groups. Heteroatoms like sulphur, nitrogen, oxygen and halogens as well as cyclic functionalities may occur. The most elementary fatty acyls are saturated, but lipids with double or triple bounds have been reported as well. A potential cycle usually consists of three to a maximum of six carbon atoms. Subgroups of fatty acyls are e.g. lactones and wax esters.[29] A representative molecule, stearic acid, can be seen in Figure 3.

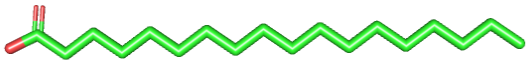


Figure 3: Stearic acid as an example for fatty acyls. Carbons are depicted in green, oxygen in red. Hydrogens have been hidden for clarity. Visualized with PyMOL.

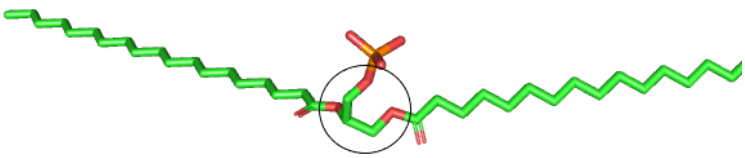


Figure 2: A representative phospholipid. Phosphor is depicted in orange; the glycerol backbone is circled in black. Visualized with PyMOL.

Lipids, that contain glycerol are divided into two groups: *glycerolipids* and *glycerophospholipids* (see Figure 4). One common group of glycerolipids consists of

esters of fatty acids with glycerol, which can be substituted at one, two or all three hydroxy groups of glycerol. Glycerophospholipids are built up of phosphates or phosphonates as esters with a hydroxyl group of glycerol. The essential "head group", which is polar, is placed at the sn-3 position of glycerol. A second glycerol (like in glycerophosphoglyceroles or glycerophosphoglycerophosphates) or even a third can be found in cardiolipins (CL). All the glycerols are part of the head group. If a lipid has another substituent on the sn-1 position than on sn-3, a chiral centre is generated at the second carbon of glycerol.[29]

Saccharolipids are built up of fatty acyls together with a sugar backbone instead of glycerol. The mentioned parts are directly linked. This group should not be mixed up with the term "glycolipid" as every other group of lipids contains glycan derivates. Saccharolipids can also be phosphorylated or appear as glycans. An example for saccharolipids is presented in Figure 5Figure 4: Lipid IVA as an example for saccharolipids. The sugar backbones are marked by circles, nitrogen is depicted in blue. Visualized with PyMOL..[29]

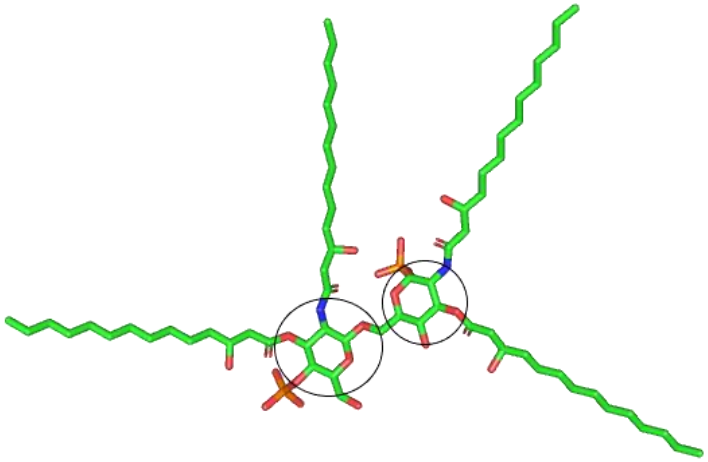


Figure 4: Lipid IV_A as an example for saccharolipids. The sugar backbones are marked by circles, nitrogen is depicted in blue. Visualized with PyMOL.

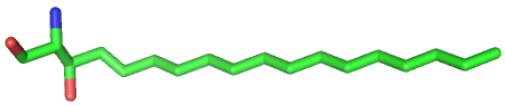


Figure 6: Sphingosine, a possible backbone of sphingolipids containing a double bond at C-4 (trans). Visualized with PyMOL.

The main part of *sphingolipids* is a long hydrophobic chain. Their synthesis requires a long-chain fatty acyl-CoA and serine. One sphingolipid backbone, sphingosine, is presented in Figure 6. For example, they can be turned into ceramides, glycosphingolipids or phosphosphingolipids. Ceramides can be found in the skin and consist of a sphingoid base with a fatty acid that is linked via an amide bond.[29] Ceramide D as an exemplary sphingolipid can be seen in Figure 7. *Polyketides* occur in microbes and plants and are therefore not described in detail, as they do not play any role in mammalians. The last two groups, *sterol* and *prenol lipids*, are both

built up of isoprene units but show elementary differences in their function and structure. The mevalonic acid pathway is essential for prenol lipids; examples for this group are ubiquinones, vitamin A (Figure 8) and E. Polyprenols are important for transporting oligosaccharides across biological membranes. Sterol lipids are divided into subgroups based on their function.[29] An example is given in Figure 9.

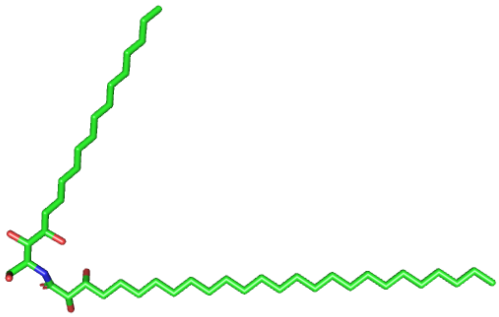


Figure 7: Ceramide D is a N-acetylated sphingolipid. Visualized with PyMOL.

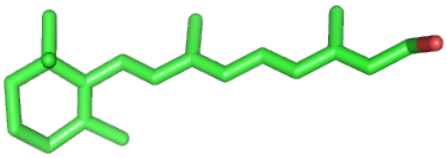


Figure 8: The isoprenoid all-trans retinol (Vitamin A) as an example for prenol lipids. Double bonds are located at C-2, C-4, C-6 and C-8. Visualized with PyMOL.

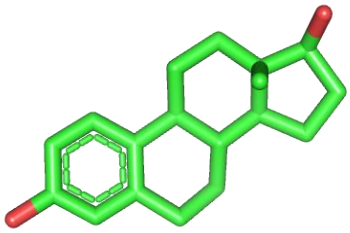


Figure 9: An aromatic sexual hormone as representative for sterol lipids, 17β-Estradiol. Visualized with PyMOL.

5.2. Biological function

Lipids play an important role in many biological processes: they count as main component of organelles like lysosomes, endosomes, the Golgi apparatus, the endoplasmic reticulum and, of course, the plasma membrane. Some lipids take part in signalling processes, others are responsible for keeping up correct structures, for example membrane curvatures. On the other hand, lipids can complete the function of many proteins as they can be bound to them in the posttranslational modification process.[30] Together with sphingomyelins and glycerophospholipids, sterol lipids are essential components of membrane bilayers. Sterol lipids play important roles as signalling

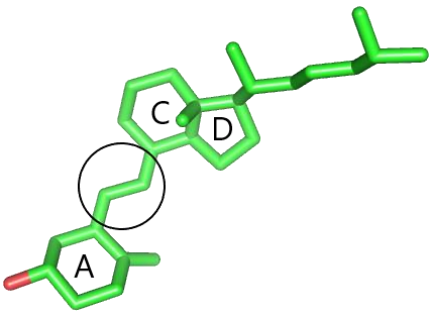


Figure 10: A visualisation of cholecalciferol. The cleavage of ring B is marked by a black circle; ring A, C and D are labelled. Visualized with PyMOL.

molecules and hormones. Depending on the number of carbons, the C_{18} steroids represent oestrogens, C_{19} androgens and C_{21} glucocorticoids, mineralocorticoids and progestogens. Vitamin D (cholecalciferol) counts as secosteroid, which means the molecule is cleft at ring B.[29] The structure of cholecalciferol is given in Figure 10.

5.3. Lipid composition of the plasma membrane

For the understanding of the function of lipids and also many proteins (i.e. K_{IR} channels), it is important to give a detailed explanation of the plasma membrane. An obvious purpose of this phospholipid bilayer is the division of two aqueous compartments. Cell-cell recognition and directed transport of ions and molecules are essential functions as well. The most common lipids in the plasma membrane are phosphatidylethanolamine (PE), phosphatidylcholine (PC), sphingomyelin (Sph) and phosphatidylserine (PS). It must be mentioned that the composition of the outer and inner leaflet of the membrane is not equal. The primarily presented lipids in the inner leaflet are PS, which is negatively charged, and PE. Phosphatidylinositol (PI) is also negatively charged and can only be found in the inner leaflet, where it represents an important part for signalling processes. As a result of the charges of the mentioned lipids, the cytosolic side of the cell shows a negative charge.[31]

On the other hand, sphingomyelin, PC and glycolipids are the important lipid groups of the outer leaflet. As they are more hydrophilic, the carbohydrate parts of glycolipids are headed towards the outside of the membrane. Cholesterol is widely distributed in both sides of the bilayer. An important property of the plasma membrane is its flexibility: proteins and phospholipids can move laterally. This characteristic highly depends on cholesterol. It is located with its hydroxy group nearby the polar heads of the phospholipids. If the membrane is exposed to low temperatures, cholesterol manages to keep up the fluidity and protects the membrane from freezing. On the other hand, the flexibility of the membrane is restricted at higher temperatures because of interactions of cholesterol with phospholipids.[31] Cholesterol can interact directly with membrane proteins or indirectly via modifications of the physical characteristics of the phospholipid bilayer. An example for a direct interaction is a pore block by cholesterol.[32] This

mechanism has been observed in large-conductance voltage/Ca^{2+}-gated K^+ channels.[33] Other mechanisms are allosteric interactions, managed by stabilizing different conformations of a protein.[32]

Although all lipids in the plasma membrane are assumed to move freely, there are some parts, called lipid rafts, which contain more sphingolipids and cholesterol than other sections and can only diffuse laterally as complete domains; lipid rafts themselves are not rigid.[31] The parts of the plasma membrane can therefore be divided into non-raft and raft domains. These microdomains are essential for cell migration, endocytosis, exocytosis and signalling processes. Membrane proteins often occur in the presence of lipid rafts, suggesting that the mentioned domains may influence the function of proteins. Cholesterol is needed to stick the lipids and proteins in each lipid raft together. Protein-protein interaction can be inhibited or enhanced by lipid rafts. For example, representatives of proteins in the same raft are more likely to interact with each other than with non-raft proteins. Lipid rafts and cholesterol in general seem to play an important role in the central nervous system.[34]

5.4. Protein lipidation

The lipidation of proteins results in different property changes. The process happens co- or posttranslational and enables proteins to change their subcellular localizations, enhance the affinity to the membrane bilayer or support proteins in their folding and stabilization process. Interactions with other proteins may also be influenced by lipidation. For example, fatty acyls can be attached to lysine, serine or cysteine and in rare cases even to a glycine at the N-terminus of a protein. The binding of myristate to glycine is irreversible in contrast to the S-palmitoylation of a cysteine residue. The latter acylation and deacylation can occur often and results in different functions like signalling processes. S-palmitoylation is important for the regulation of the function of immune receptors, cellular junction proteins, kinases and receptors as well as transcription factors. Another form of lipidation is called prenylation and describes the mostly irreversible binding of isoprenoids to proteins. In this case, farnesyl- and geranylgeranyl groups are added to a cysteine as it occurs in the Ras family. Again, the process changes the affinity of the

affected proteins to the plasma membrane. A modification by cholesterol is quite rare but has been reported in Hedgehog proteins.[35]

Although phospholipids are important molecules for signalling processes, they are usually not attached to proteins except for one example: PE is bound to Atg8/LC3 – a protein responsible for the autophagy process.[35]

Glycosylphosphatidylinositols are known to act as an anchor for many proteins after their synthetization in the ER. These complex glycolipids are usually bound to the C-terminus and it is estimated that about one per cent of eukaryotic proteins show this posttranslational modification.[35]

5.4.1. Palmitoylation of a cysteine residue in $K_{IR}6.2$

The S-palmitoylation of a cysteine residue in $K_{IR}6.2$ subunits is associated with an increase in channel opening probability. This phenomenon has been observed in a recent study by Yang et al.[36] This reversible process is mediated by palmitoyl acyltransferases via palmitoyl-CoA. The opposite effect is called depalmitoylation, which is managed by acyl-protein thioesterases called APT1 and APT2. SUR subunits are not involved in this process. Neither the channel surface expression nor trafficking processes are affected by the palmitoylation. The lipidation was experimentally induced by either adding palmitic acid or by inhibiting thioesterases. $K_{IR}6.2$ has seven cysteine residues, which are well conserved among $K_{IR}3$ and $K_{IR}6$ channels. Therefore, it is important to determine the cysteine residue responsible for the regulation process. Cys166 has been found to be the most probable candidate, as it is located nearby the inner membrane interface. Therefore, Cys166 was changed to valine, which prevented the channel from being palmitoylated. The lipidation of $K_{IR}6.2$ seems to influence the sensitivity to PIP_2. To provide evidence for this phenomenon, a rundown of $K_{IR}6.2$ was induced with Ca^{2+}, which is known to cause a hydrolysis of PIP_2 via the activation of phospholipase C. Afterwards, PIP_2 with defined C-8 acyl chains was added and the channels slowly recovered. It has been observed that the recovery is faster, when thioesterases are inhibited or palmitic acid is added beforehand. This effect did not occur in the C166V mutant, which leads to the conclusion, that the palmitoylation of Cys166 causes a higher PIP_2 sensitivity.[36]

6. The role of cholesterol in K_{IR} channels

6.1. The effect on K_{IR} channel activity

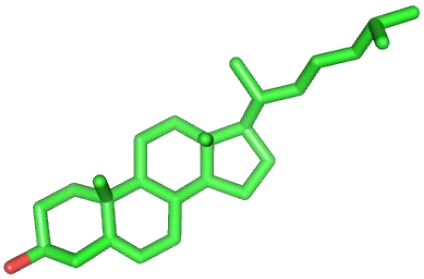


Figure 11: A visualisation of cholesterol. The hydroxy group at position 3 is depicted in red. Visualized with PyMOL.

What has been described earlier can be applied to inward rectifier potassium channels as well: cholesterol can not only cause changes in physical properties concerning the plasma membrane, but also bind directly to channels, leading to specific modulations. The chemical structure of cholesterol is visualized in Figure 11. The interaction of cholesterol with K_{IR} channels has mainly an impact on the cardiovascular system and has been reported in every group except the $K_{IR}5$ family.[32] A study by Romanenko et al. describes the impact of cholesterol on $K_{IR}2.x$ current density in aortic endothelial cells.[37] The mainly expressed channels in the mentioned cells are $K_{IR}2.1$ and $K_{IR}2.2$.[38] For the experiments, they altered the cholesterol composition of the lipid bilayer. For one investigation they changed about half of the native cholesterol to epicholesterol, an optical isomer with an altered rotational angle of a hydroxy group (located on position 3, see Figure 11), which led to an increase in current density. On the other hand, an enrichment of cholesterol caused a reduction. An optical isomer is often used in investigations like the mentioned experiment to widely maintain the natural physical properties but to enable the recognition of different biological activities. It has been demonstrated that the change to epicholesterol does not have significant impacts on the membrane fluidity. Romanenko et al. suggest that the composition of the plasma membrane (concerning cholesterol) alters the numbers of active K_{IR} channels, as their open probability and conductance do not show any changes.[37]

6.1.1. Silent channel hypothesis

A possible explanation for the mentioned phenomenon is the “silent channel” hypothesis. It has been shown that the expression of $K_{IR}2.1$ does not depend on cholesterol.

Furthermore, inhibited protein synthesis does not have any effects on the conductance caused by a depletion of cholesterol. It is suggested that $K_{IR}2.x$ channels exist in two different states, namely active and silent channels. Silent channels are stable in the closed state while active channels are more likely to change from the closed to the open state. Cholesterol is thought to influence these states of K_{IR} channels and therefore the closed state occurs more often in lipid rafts. On the other hand, a depletion or excess of cholesterol does not alter the distribution of K_{IR} channels to raft or non-raft domains much. These findings lead to the suggestion that the composition of lipid rafts is more important for the activity of K_{IR} channels than their location per se.[39] Another result is that the impact of cholesterol on inward rectifier potassium channels is enantioselective.[37] As the effect on membrane properties usually does not depend on its different isomeric forms, it seems reasonable that the effect on K_{IR} channels results from direct sterol-protein interactions. Therefore, several experiments concerning possible binding sites have been performed, which are described in detail later.[40]

6.2. Cholesterol in $K_{IR}3$ channels

A study by Bukiya et al. concentrates on the role of cholesterol in $K_{IR}3$ channels located in the hippocampus.[41] Concerning the brain, GIRK channels are important in the regulation process concerning the excitability of neurons. A higher channel activity causes less neuronal excitability and vice versa. The most important K_{IR} channel in the brain is GIRK2. An *in vivo* experiment included rats, which were given a diet that was high in cholesterol. The examined cells were neurons isolated from the hippocampus. Interestingly, higher cholesterol levels could be found even in the brain, although here cholesterol is only synthesized newly and not transferred through the blood brain barrier. A possible explanation for this phenomenon could be that unphysiologically high levels of blood cholesterol may damage the blood brain barrier.[41]

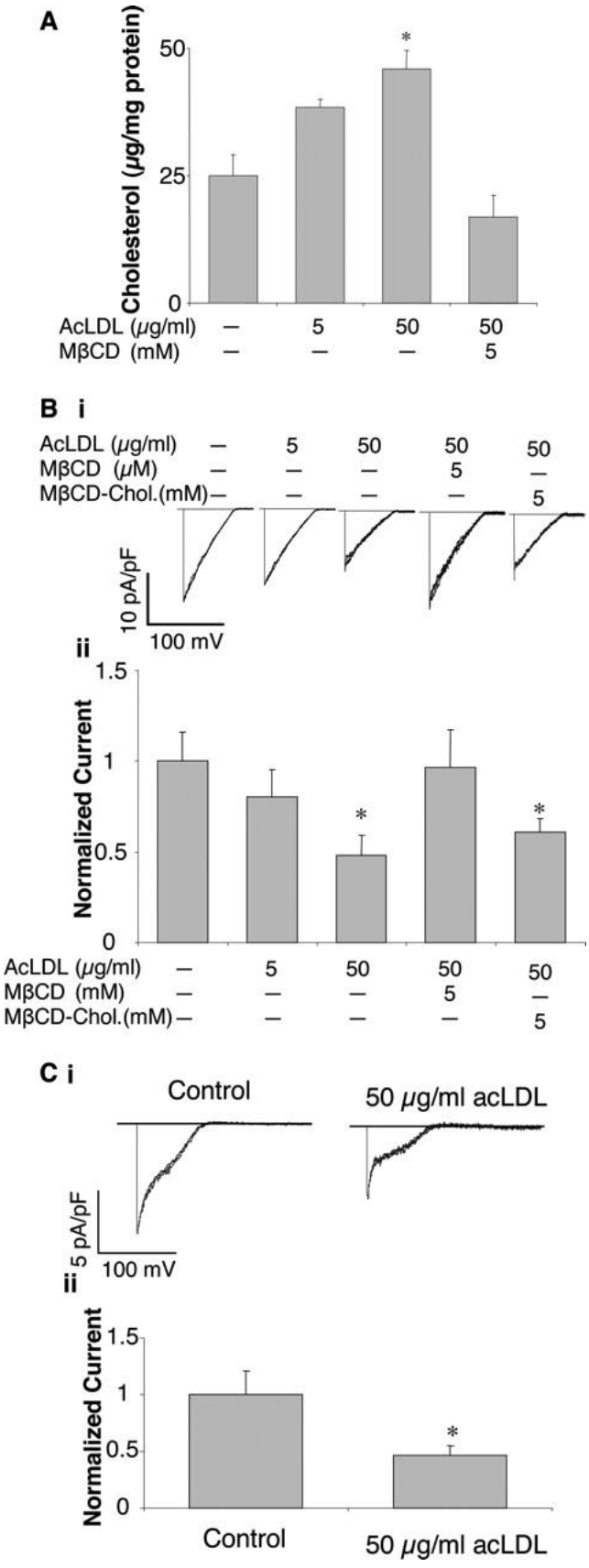
In an *in vitro* experiment, pyramidal neurons from the hippocampus were enriched with cholesterol by using MβCD as carrier for cholesterol. One outcome was that cholesterol regulated $K_{IR}3$ channels up as the cells showed increased currents. On the other hand, currents decreased upon a cholesterol reduction.[41]

Furthermore, a mutated GIRK2 channel was examined. Therefore, the mutation E152D was introduced, which causes a higher number of GIRK2 channels to be expressed on the surface and enhances their activity. To control the lipid concentration surrounding the $K_{IR}3.2$ channels, planar lipid bilayers were used. It was shown that the measured currents resulted from K_{IR} channels, since the addition of tertiapin-Q, a selective GIRK blocking agent, caused a significant decrease in channel currents. Interestingly, adding cholesterol to the lipid bilayers containing the mentioned mutated GIRK2 channels, increased the open probability notably about 21-fold in comparison to bilayers without cholesterol. However, the unitary conductance was not influenced significantly. Since the impact of cholesterol on $K_{IR}3$ channels differs from the way $K_{IR}2$ channels react towards a cholesterol enrichment, possible binding sites in $K_{IR}3$ were analysed. The results shall be described later (see chapter 6.8).[41]

6.3. Cholesterol in bacterial K_{IR} channels

It has been revealed that cholesterol has the same effects in bacterial K_{IR} channels as in eukaryotic channels. The inhibition is enantioselective in K_{IR}Bac1.1 and K_{IR}Bac3.1 as well as in human $K_{IR}2.x$, which has been shown in a study by Fürst et al.[40] One equivalent cholesterol binding site to human $K_{IR}2.2$ has been discovered in K_{IR}Bac1.1, although cholesterol does not bind in the same way. In comparison of the two mentioned channels, cholesterol is rotated 180° concerning the rough face. The tail of cholesterol reaches into a different groove, which contains the residues Phe132, Val133, Ser136 and Gly137. Epicholesterol could not bind to this site at all or only with high energies due to steric clashes. Although the binding site seems to be conserved among eukaryotic and prokaryotic cells, it is important to mention, that cholesterol does not occur in bacterial membranes.[40]

6.4. In vivo studies



A study by Fang et al. considers *in vivo* aspects as well.[38] They used cell cultures for the *in vitro* part and a pig model to demonstrate K_{IR} suppression by very low-density lipoproteins (VLDL) and acetylated low-density lipoproteins (acLDL). For the *in vitro* experiment, aortic endothelial cells (HAEC) were exposed to acLDL and VLDL, leading to increased cholesterol levels. This caused a suppression of K_{IR} currents. The reversibility of this process was proven by adding methyl-β-cyclodextrin (MβCD), which depletes the excess of cholesterol. A treatment of cells with VLDL results in different membrane potentials (a shift to more positive) and more depolarizations. Results of the acLDL treatment are presented in Figure 12; the effects of VLDL were similar.[38]

Figure 12: A shows the amount of cholesterol inside HAECs after a 24-hour treatment with 5 or 50µg/mL acLDL or with an addition of MβCD after a 50µg/mL cholesterol treatment in comparison to a standard. B depicts the normalized currents after each treatment. C presents the sensitivity of K_{IR} channels after a 24-hour treatment of 50µg/mL acLDL. High K^+ concentrations were used in B, physiological concentrations in C. Taken from Fang et al., 2006.[38]

Cells exposed to an oxidized modification of LDL did not show any significant changes in the functions of K_{IR} channels.[38]

To investigate the *in vivo* part of the study, pigs have been given a cholesterol rich diet leading to higher LDL and plasma cholesterol levels. Furthermore, it has been proven that more cholesterol has been embedded in endothelial cells caused by the diet. Hypercholesteraemic pigs showed decreased K_{IR} currents *ex vivo*. Similar to what has been observed in cell culture experiments, currents could be normalized by adding MβCD to the isolated aortic endothelial cells. As a result of both *in vitro* and *in vivo* experiments, the mentioned study provides evidence for the hypothesis, how hypercholesterolaemia reduces the current of K_{IR} channels and therefore leads to endothelial dysfunction.[38]

6.5. Cholesterol sensitivity

An overview of the effects of cholesterol on different K_{IR} channels is given in Table 4.

Channel	Activity with cholesterol	References
$K_{IR}1.1$	Suppression	Rosenhouse-Dantsker et al., 2010[42]
$K_{IR}2.x$	Suppression	Levitan, 2009[39] Rosenhouse-Dantsker et al., 2010[42]
$K_{IR}3.x$	Enhancement	Rosenhouse-Dantsker et al., 2010[42] Bukiya et al., 2017[41]
$K_{IR}4.1$	Suppression Depletion leads to less functional channels	Rosenhouse-Dantsker et al., 2010[42] Levitan, 2009[39]
$K_{IR}6.x$	Effect of K_{ATP} blockers → increases → is not altered	Mathew and Lerman, 2001[43] Genda et al., 2002[44]
$K_{IR}7.1$	Enhancement	Rosenhouse-Dantsker et al., 2010[42]
$K_{IR}Bac$	Suppressed	Fürst et al., 2014[40]

Table 4: How K_{IR} channels react towards cholesterol with corresponding references.

6.5.1. Decreased channel activity

Not every inward rectifier potassium channel shows the same reaction and sensitivity to cholesterol. A comparison of $K_{IR}2.x$ channels shows that $K_{IR}2.1$ and $K_{IR}2.2$ are more sensitive than $K_{IR}2.3$, which is about 50% sensitive. The least sensitivity has $K_{IR}2.4$. Some channels like $K_{IR}2.1$ are more likely to be associated with lipid rafts than others (i.e. $K_{IR}2.3$). A connection between the sensitivity to cholesterol and K_{IR} channels located in lipid rafts seems reasonable. $K_{IR}4.1$, $K_{IR}3.1$ and $K_{IR}3.2$ are associated with lipid rafts. Concerning a fusion protein of $K_{IR}3.1$ and $K_{IR}3.2$, it has been shown that a block of cholesterol synthesis by lovastatin does not affect the function of the channel. As G-proteins, like they occur with $K_{IR}3.x$ channels, are known to be located in lipid rafts more likely, it seems reasonable that the regulation of Gβγ proteins depends on the composition of lipid rafts.[39] $K_{IR}6$ channels remain controversial concerning their reaction towards altered cholesterol concentrations. They could be activated or inhibited by a cholesterol enrichment.[42]

6.5.2. Increased channel activity

Interestingly, cholesterol has the opposite effect on some other K_{IR} channels. GIRK channels react with an upregulation upon cholesterol enrichment.[41] The reaction of $K_{IR}4.1$ towards cholesterol enrichment is seen somehow controversially in different publications. A study by Rosenhouse-Dantsker et al. suggests that $K_{IR}4.1$ currents are suppressed by cholesterol.[42] However, Levitan writes how a depletion of cholesterol leads to a loss of function in $K_{IR}4.1$.[39] The process is reversible, as the function is restored by adding cholesterol again. $K_{IR}4.1$ and the co-assembly with $K_{IR}5.1$ are associated with raft and non-raft domains. There are different approaches of explanations for these differences between single K_{IR} channels.[39]

6.5.3. PIP_2 in cholesterol-rich parts

One possible reason for this difference is the localization of PIP_2 in parts that are rich in cholesterol. In the case of a cholesterol depletion, channels dissociate from PIP_2 and therefore lose an important activation factor. As $K_{IR}2.x$ channels are also dependent on PIP_2, this explanation does not constitute conclusive evidence.[39]

6.5.4. Localisation in lipid rifts

A more plausible explanation is the difference in the localization of different K_{IR} channels. $K_{IR}2$ channels colocalize with caveolin in caveolae (invaginations of the plasma membrane, which are flask-shaped) in contrast to $K_{IR}4.1$.[1,39] A depletion of cholesterol does not alter the distribution of $K_{IR}2.x$ between non-raft and raft domains. In contrast, $K_{IR}4.1$ seems to disappear from lipid raft domains. It can be assumed that members of the $K_{IR}2$ family are located at different parts of the plasma membrane and therefore their association with domains that are enriched with cholesterol is more stable.[39]

6.6. Cholesterol and caveolin-1

A study by Han et al. examined the connection between the $K_{IR}2.x$ sensitivity to cholesterol and caveolin-1 (cav-1).[45] Caveolins in general are key proteins in the centre of caveolae.[46] An inhibition of $K_{IR}2$ currents by cholesterol has been shown in purified channels without any native lipids and caveolin around. Cholesterol is presumed to interact directly with K_{IR} channels. On the other hand, the cholesterol sensitivity may also at least partially result from their caveolin interaction. The absence of cholesterol leads to disruptions of lipid rafts and caveolae and therefore to dissociated caveolins. A co-expression of $K_{IR}2.1$ and cav-1 showed a decrease in whole-cell currents but did not alter the surface expression of $K_{IR}2.1$. Like the effect of cholesterol on K_{IR} channels, cav-1 does not influence the open probability as well as unitary conductance. Furthermore, cav-1 is not necessary for the $K_{IR}2$ sensitivity to cholesterol but shifts it, when cav-1 is

overexpressed (see Figure 13). The regulative mechanism of both cholesterol and cav-1 seems to be similar.[45]

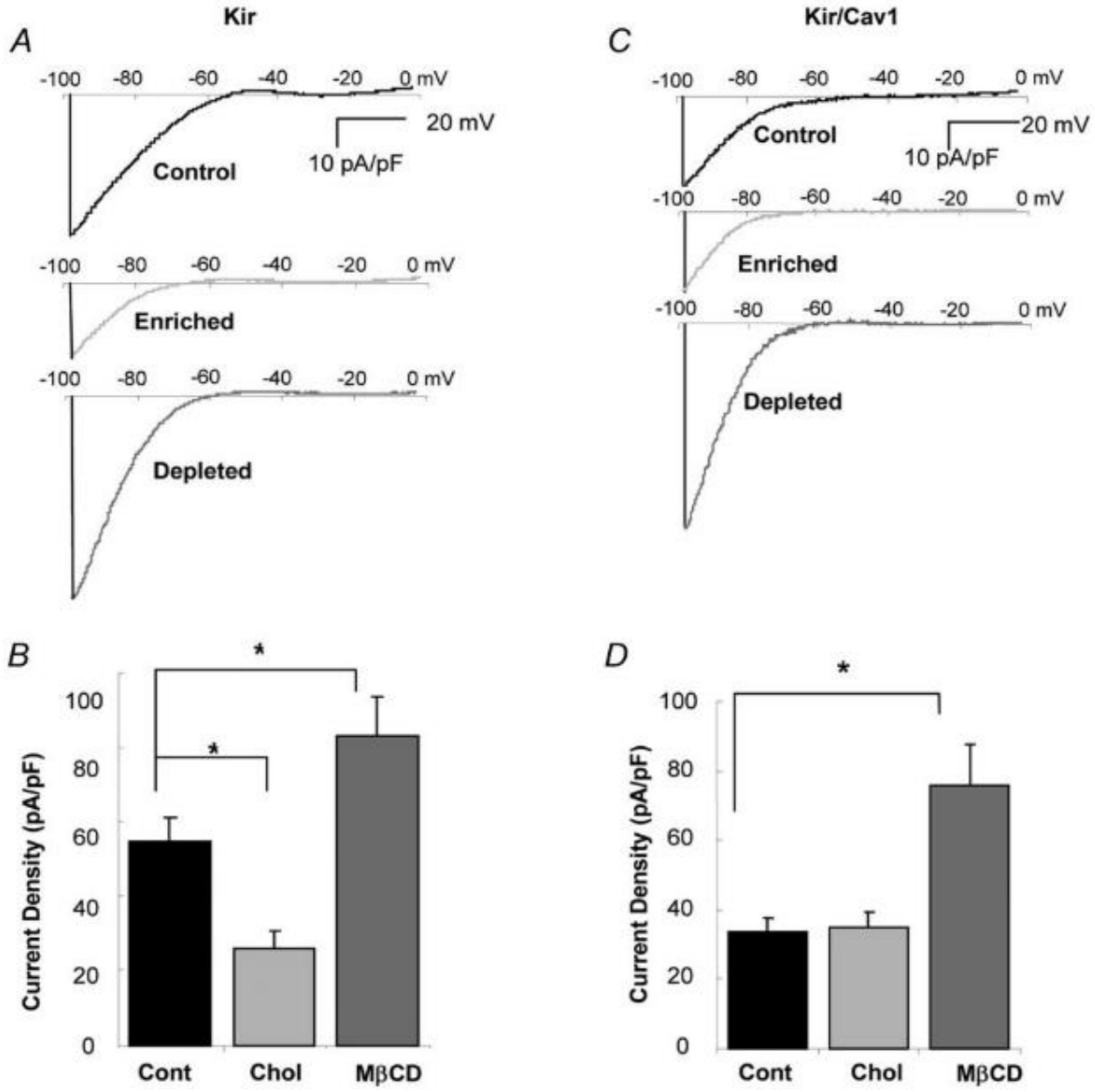


Figure 13: Left: $K_{IR}2.1$ co-expressed with green fluorescent protein (GFP) in HEK cells, right: $K_{IR}2.1$ co-expressed with cav-1 and yellow fluorescent protein (YFP). The experiments were performed with control cells (Cont), cholesterol enriched cells (Chol) and cholesterol depleted cells (MβCD). A and C show current traces of the used HEK cells, B and D average peak current densities (-97mV). Whole cell currents decrease when $K_{IR}2.1$ is expressed with cav-1 in general, a shift in cholesterol sensitivity by cav-1 can be seen: the effect of cholesterol enrichment is weaker with cav-1; on the other hand, the effect of a depletion of cholesterol is increased. Taken from Han et al., 2014.[48]

6.7. Putative cholesterol binding sites in $K_{IR}2$ channels

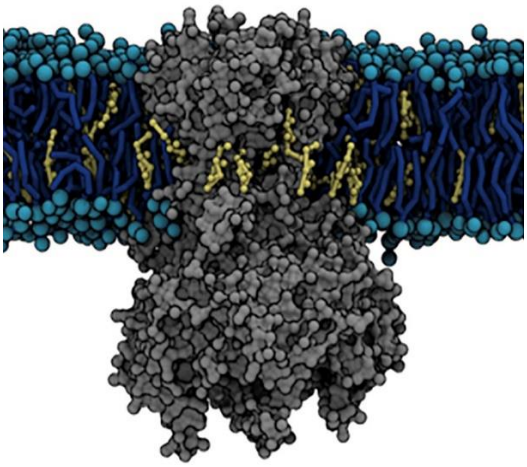


Figure 14: A model of chicken $K_{IR}2.2$ (grey) embedded in a lipid bilayer (blue) with several cholesterol molecules binding to the part, that is exposed to the membrane. Taken from Barbera et al., 2018.[48]

Typical cholesterol binding motifs have been identified in diverse channels and receptors like in BK channels or transient receptor potential vanilloid 1 (TRPV1) channels and even in acetylcholine receptors (AChR). The known motifs that occur in the mentioned channels and receptors are the cholesterol recognition amino acid consensus motif (CRAC) and CARC, its inverted sequence. Another sequence is the cholesterol consensus motif (CCM). All these sequences are not reported in K_{IR} channels except for $K_{IR}2.1$ on a cytoplasmic part, which shows a CRAC motif. However, it does not seem to play an important role, as this amino acid sequence is energetically unfavourable for the binding of a cholesterol molecule.[47] Docking studies have been performed by Barbera et al. to show possible cholesterol binding sites.[48] An overview of cholesterol binding to $K_{IR}2.2$ in general is shown in Figure 14. This study concentrated on a coarse grained (CG) molecular dynamic

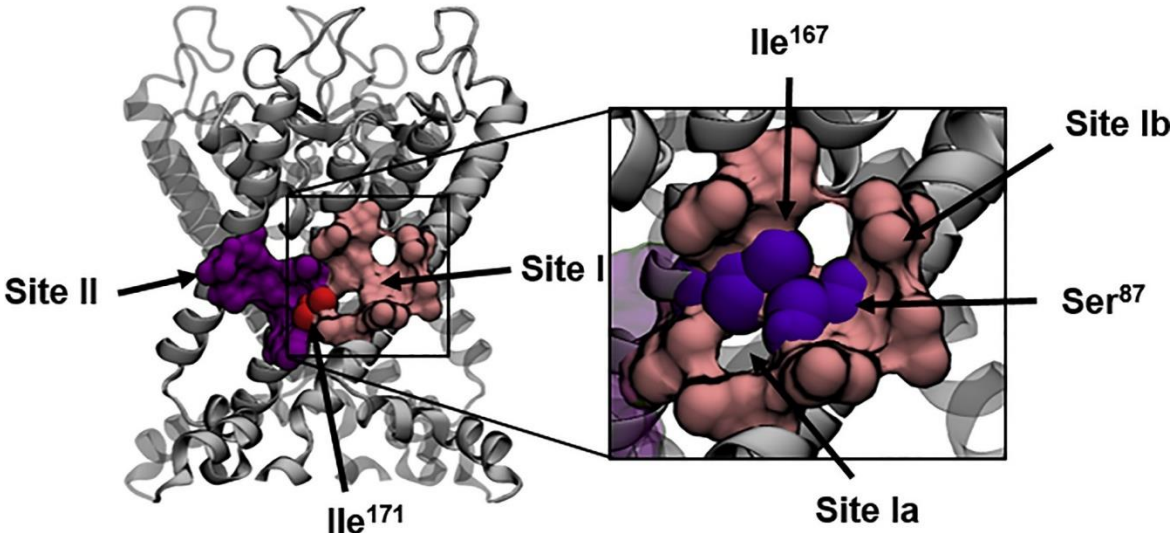


Figure 15: A model of cholesterol binding sites I and II in chicken $K_{IR}2.2$. They share isoleucine at position 171. The closeup shows site I more in detail with its subsites Ia and Ib, which share serine (87) and isoleucine (167). Taken from Barbera et al., 2018.[48]

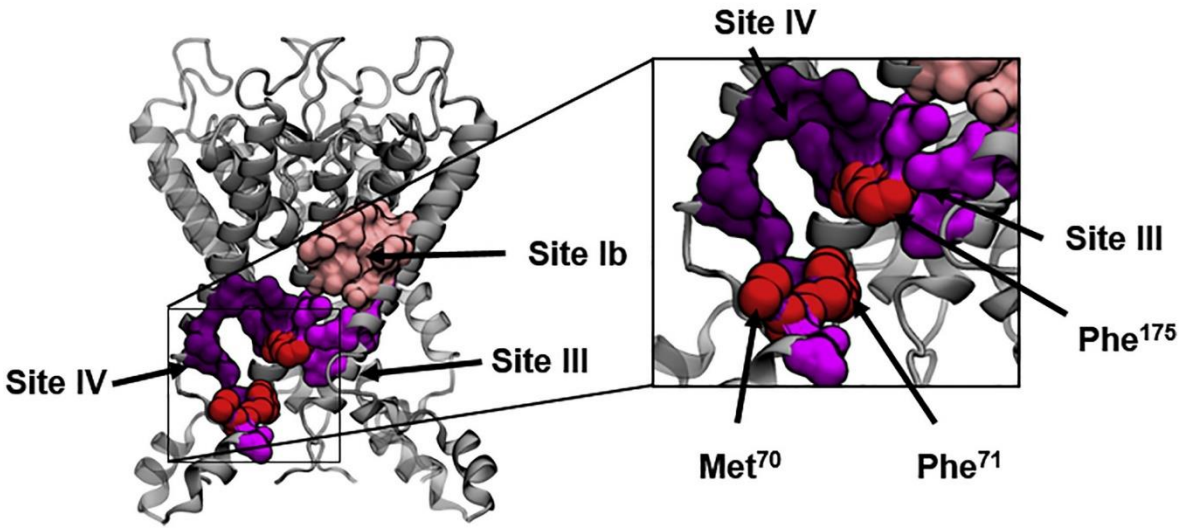


Figure 16: The location of site Ib, III and IV in the closed state (chicken $K_{IR}2.2$). The detailed snapshot on the right shows three amino acid residues shared by site III and IV (Met70, Phe71 and Phe175). Taken from Barbera et al., 2018.[48]

simulation, which especially considers lipids surrounding the protein of interest. Multiple putative binding sites for cholesterol have been identified in chicken $K_{IR}2.2$ channels. The ones found in the open state are different to the ones in the closed state. Models of the crystal structure in both open and closed state (two different simulations) from the homotetrameric $K_{IR}2.2$ channel have been embedded in a simulated lipid bilayer containing a phospholipid (1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine, POPC) and 30% cholesterol. The chosen amount of cholesterol typically occurs in mammalian plasma membranes. It has to be mentioned, that two different crystal structures were used, which both have closed helix bundle crossing (HBC) gates; only the G-loop gate conformation differs. Around 22 molecules have been found to bind to $K_{IR}2.2$ in this simulation in the open state and 24 in the closed. The interactions range from transient to persistent and are located at the surface or in hydrophobic pockets in the transmembrane helix. A reduction of cholesterol led to a decrease of its occupancy at many sites except for some discrete sites with a higher affinity. At a 15% cholesterol concentration, only eleven molecules showed contact with $K_{IR}2.2$ with a decrease in contact duration. To investigate the localization of more important sites, amino acid residues with a long contact time have been observed especially in the transmembrane domain. Therefore, the region from position 60 to 190 has been investigated, because the pore, the transmembrane helix and slide helices are covered within this area. The mentioned study

describes four specific binding sites (I-IV), their visualisation is presented in Figure 15 and 16.[48] Binding site I is located between two $K_{IR}2.2$ subunits and shows higher occupation rates. Important residues are L83, S87, I167, C170 and I171 (Ia) as well as L84, S87, L88, L91, V163, V164 and I167 (Ib). Site II lies within one subunit between the two α-helices TM1 and TM2 and contains the residues A89, V92, S93, V168, I171, I172 and F175. Both sites occur in the open state. Site III contains residues M70, L83, L84, F86, S87, F90, C170, F175, M176 as well as I180 and can be found between two subunits. Site IV is located at only one subunit nearby the slide helix and important residues are M70, F71, C74, M82, L85, A89, I172, F175 and M176. Ib is the only subsite that occurs in the open and closed state. Single residues are shared in the closed and open state like Leu83, Ser87 and Cys170 (site Ia and III) or Ala89, Ile172 and Phe175 (site II and IV). To enable a better overview of the locations of each site, Ia is compared with III and II with IV. The simulation enables a possible explanation of the influence by cholesterol on K_{IR} channels: if cholesterol binds to site III or IV, the closed state is more stable than by the binding to I or II. As the locations of I and III as well as II and IV do not differ much, cholesterol might wander from the sites of the open state to the ones of the closed state, resulting in the stabilisation of the closed state. On the other hand, a low cholesterol level leads to a loss in molecules bound to $K_{IR}2.2$ especially at site Ib and II and the open state is more favoured. Figure 17 depicts cholesterol binding site Ia, II, III and IV in comparison. It is obvious that their localizations (Ia and III, II and IV) are quite similar.[48]

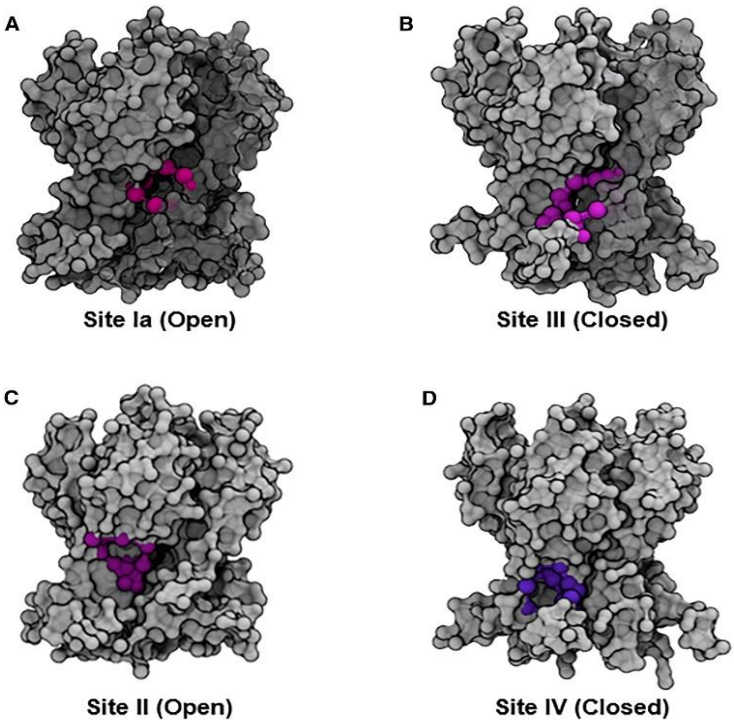


Figure 17: A comparison of the locations of cholesterol binding sites Ia-IV in $K_{IR}2.2$. Taken from Barbera et al., 2018.[48]

6.7.1. Mutagenesis studies concerning key residues

A study by Fürst et al. suggests the following key residues in cholesterol binding pockets in $K_{IR}2.1$: Ser95 and Ile171 (equivalent to Ser94 and Ile172 in human $K_{IR}2.2$, Ser93 and Ile 172 in chicken $K_{IR}2.2$).[40] A hint for the importance of these residues in the binding site is given by $K_{IR}7.1$, which has histidine (on position 67) and leucine (148) on the equivalent positions and shows different effects upon cholesterol depletion. These two residues do not occur in any other K_{IR} channels, which have serine and isoleucine at the equivalent positions, except for the $K_{IR}6$ family.[40] The channel function of $K_{IR}7.1$ is hardly affected by reduced cholesterol levels and presumed to be even enhanced upon an enrichment.[42] In a docking study concerning cholesterol in $K_{IR}7.1$ at the equivalent binding site of $K_{IR}2$ channels, His67 and Leu148 caused steric clashes. The histidine residue has been found to be unable to rotate enough for avoiding a clash. This finding suggested further investigations of mutations in $K_{IR}2.1$, which is naturally sensitive to cholesterol.

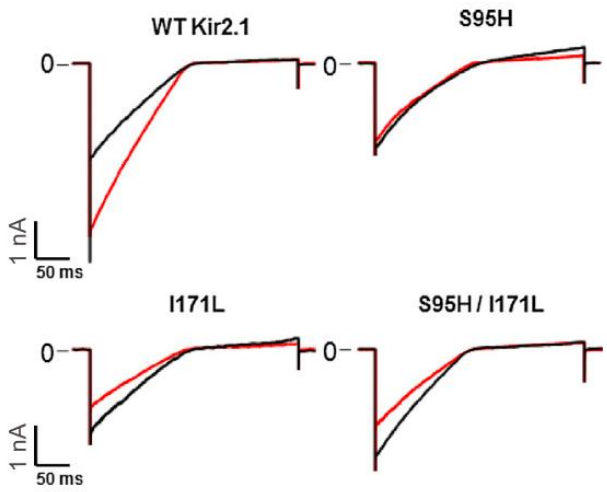


Figure 18: Patch clamp recordings of wild type (WT) $K_{IR}2.1$ and its mutations S95H, I171L and the double mutant. The black line shows recordings before and the red line after cholesterol depletion. The least difference can be seen in the S95H mutation. Taken from Fürst et al., 2014.[62]

Electrophysiological experiments were performed. Therefore, the mutations S95H, I171L and a double mutation (S95H/I171L) were introduced. Adding MβCD to the wild type $K_{IR}2.1$ led to a cholesterol depletion and thus, as expected, increased currents. The S95H mutant was insensitive to the decreased cholesterol levels, whereas I171L and the double mutant still showed a channel activation. This phenomenon may be explained by alterations of membrane properties like fluidity and thickness, caused by lower cholesterol concentrations. Cell-attached recordings were used for all variants except for the S95H mutant, where a whole-cell recording was chosen. The results are presented in Figure 18. Despite the small difference in the channel activity between

untreated and MβCD treated cells in I171L and the double mutant, the results provide evidence for the importance of the identified cholesterol binding pocket.[40]

6.7.2. The function of the CD-loop

Rosenhouse-Dantsker et al. suggest that the CD-loop plays an important role in the cholesterol binding sensitivity.[42] Independent on whether a channel is activated or inhibited by cholesterol, mutations of key residues in the CD-loop cause a loss in cholesterol sensitivity. For electrophysiological studies, Leu222 was mutated to isoleucine in $K_{IR}2.1$ and Ile229 was changed to leucine in a mutated $K_{IR}3.4$ channel. The mutation S143T in $K_{IR}3.4$ was necessary to provide functional homomeric channels. The study was performed on Xenopus laevis oocytes and recordings were taken with whole cell configurations. A decrease in normalized currents was observed in the wild type $K_{IR}2.1$ channel by adding cholesterol. The mutant still showed decreased currents, however, the shift was strikingly smaller. Similar results were achieved in $K_{IR}3.4$: although the wild type and the I229L mutant showed increased currents after a cholesterol enrichment, the shift was smaller.[42]

6.8. Putative cholesterol binding sites in $K_{IR}3$ channels

Since GIRK channels react in a different manner than $K_{IR}2$ channels towards cholesterol enrichment, putative binding sites were examined. Bukiya et al. identified two possible binding sites that match in large part with the ones found in $K_{IR}2.1$ (see alignment, chapter 6.10).[41] Involved residues in rat $K_{IR}3.2$ are V99 and L174 (binding site one, corresponding to V97 and L172 in human $K_{IR}3.2$) as well as V101 and V183 (site two, V99 and V181 in human $K_{IR}3.2$). They are presumed to interact with cholesterol directly. Another important residue, L86 (L84 in human $K_{IR}3.2$) in binding site two may be involved indirectly in the binding process as it interacts with a phenylalanine at position 186 (184 in human $K_{IR}3.2$). Latter residue is in direct contact with cholesterol. Furthermore, I195 (I193 in human $K_{IR}3.2$) is also a part of the binding site two, although a replacement does

not result in striking changes in channel cholesterol sensitivity. A mutation of M100 (M98 in human $K_{IR}3.2$) does not alter the reaction of GIRK channels towards cholesterol as well. This residue is involved in the binding site but turns away from it. Mutagenesis studies were performed to assess whether the mentioned residues are crucial for the cholesterol binding of E152D mutated GIRK2 channels. As expected, I195M and M100L did not affect the cholesterol sensitivity, whereas V99I, L86V, V101A, L174V and V183I altered it. The location of the mentioned residues in $K_{IR}3.2$ are presented in Figure 19. In conclusion, the cholesterol binding sites of $K_{IR}2.1$ and $K_{IR}3.2$ are widely comparable (see also alignment, chapter 6.10).[41]

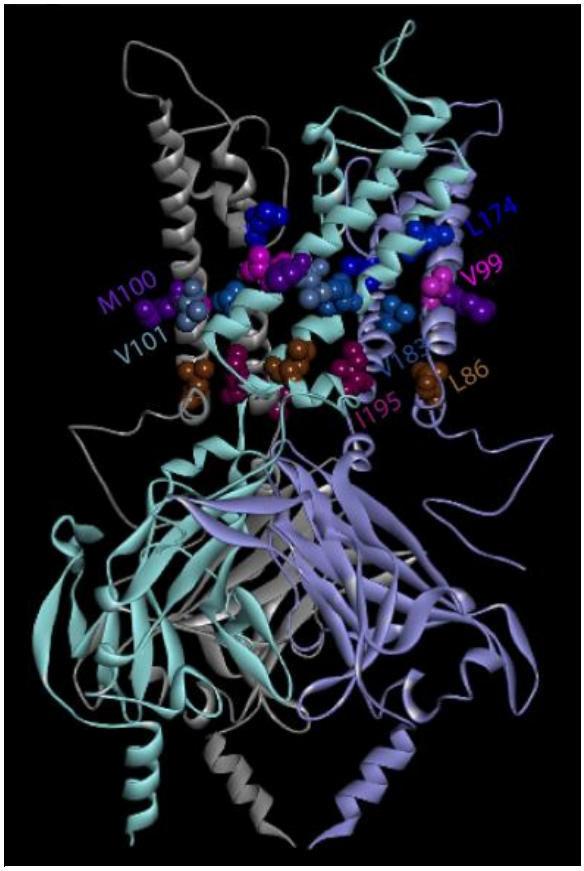


Figure 19: The location of residues that are involved in the cholesterol binding process. Binding site one contains V99 and L174, binding site two consists of residues L86, V101, V183 and I195. M100 faces away from the cholesterol binding site. Taken from Bukiya et al., 2017.[41]

Another study by Kalyani Mathiharan et al. concentrated on the conformation of GIRK2 channels depending on cholesterol and PIP_2 molecules binding to the channel.[49] They used cryogenic electron microscopy (cryoEM) of $K_{IR}3.2$ channels in the apo state and in the presence of PIP_2, cholesterol or its hemisuccinate (CHS). The most important finding of this study is that GIRK2 channels seem to be stabilized in their PIP_2 bound state by cholesterol or cholesteryl hemisuccinate, respectively. Furthermore, a flux assay using purified $K_{IR}3.2$ channels showed that the channel activity was dependent on PIP_2 and cholesterol. In comparison, channels reacted with a higher activity when they were embedded in liposomes containing CHS or cholesterol and PIP_2. In detail, the percentage of potassium ion flux was 44% with PIP_2, 65% with additional CHS and 69% with additional cholesterol.[49]

To determine the structural effects of these phenomena, cryoEM data of mouse $K_{IR}3.2$ were analysed, which widely show similarities to previously revealed X-ray structures. Nearby the PIP_2 binding site, CHS molecules could be identified. An arginine at position 92 (90 in human $K_{IR}3.2$) interacts with the hydrophilic head group of one CHS molecule, whereas the lipophilic part is surrounded by the TM1 helix residues Phe93, Leu95, Leu96 and Val99 (91, 93, 94 and 97 in human $K_{IR}3.2$) as well as the TM2 helix residues Ile175, Val178 and Leu179 (173, 176 and 177 in human $K_{IR}3.2$). Furthermore, a second CHS molecule fits in a binding pocket containing Val72, Leu79, Ile82, Leu86, Leu89, Ile97, Val101 as well as Phe186. Additionally, PIP_2 binding sites alone and in presence of

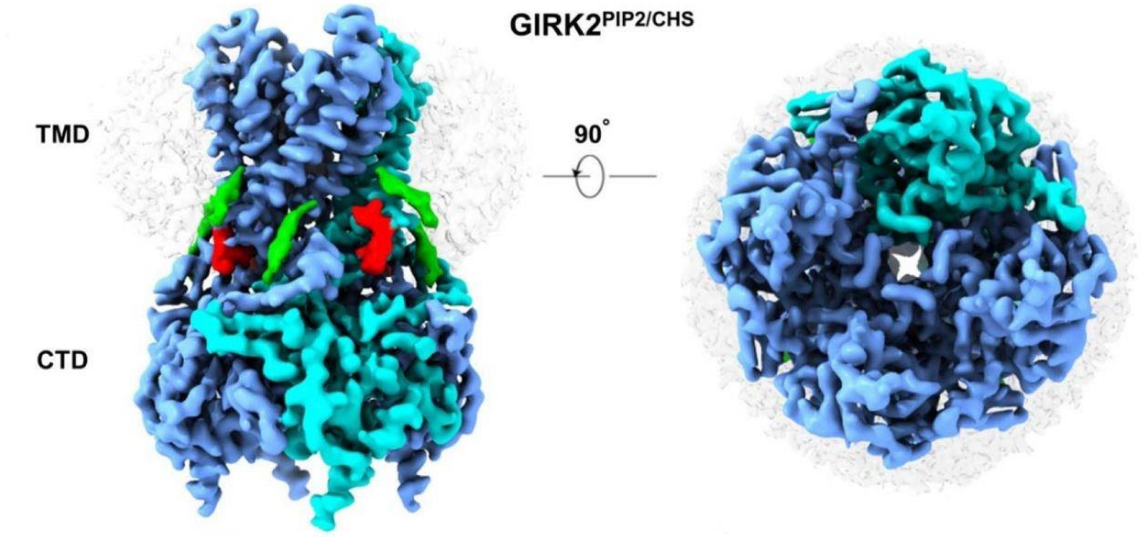


Figure 20: Left: A visualisation of GIRK2 with bound PIP_2 (red) and CHS molecules (green). The transmembrane domain (TMD) and the cytoplasmic domain (CTD) are marked. Right: GIRK2 turned 90°, a view from the bottom. Taken from Kalyani Mathiharan et al., 2020.[49]

cholesterol or CHS were analysed. The phosphate of PIP_2 on position 1' interacts with an arginine at position 92 and the 5' phosphate is in contact with three lysines at positions 194, 199 and 200 (192, 197 and 198 in human $K_{IR}3.2$). Furthermore, the carbonyl oxygen atom from the amide of Trp91 (position 89 in human $K_{IR}3.2$) is involved. The location of PIP_2 and CHS in GIRK2 channels is shown in Figure 20.[49]

The arrangement of PIP_2 alone in its binding site in $K_{IR}3.2$ is quite similar to a simulation with CHS, but more occupying molecules could be observed in the presence of CHS. A striking difference between cryoEM and X-ray data lies within the channel configuration of apo GIRK2: X-ray data suggest that the cytoplasmic domain is not that far away from the transmembrane domain than seen in cryoEM experiments. Latter show a

detachment of the mentioned domains with a spatial difference from about 23 to 28Å. The analysed apo GIRK2 channel represents a closed state conformation of the channel. Even in the PIP_2 bound state, several observed channels displayed the same detachment. Interestingly, four slightly different channel configurations could be observed with a PIP_2 molecule bound, of which three are illustrated in Figure 21. However, less channels with the mentioned spatial difference were seen in the presence of CHS, suggesting that cholesterol plays a stabilizing role in the engagement of the cytoplasmic domain towards the transmembrane domain.[49]

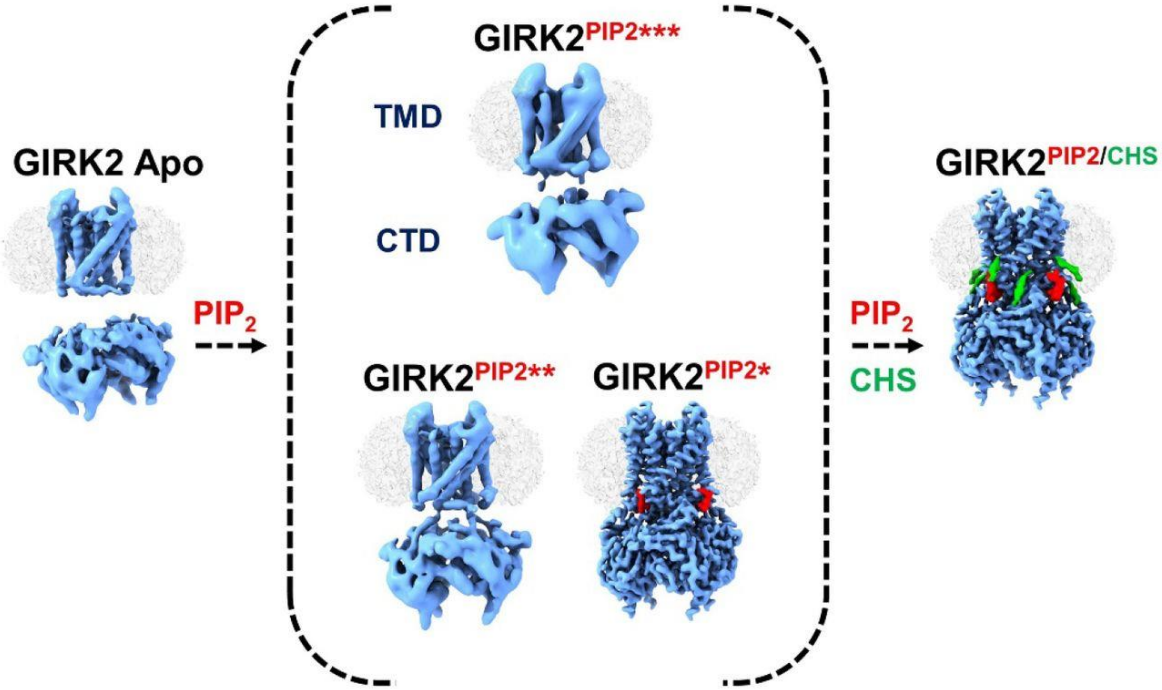


*Figure 21: Different conformations of GIRK2. The illustration on the left shows GIRK2 in its apo state. The huge spatial distance between the cytoplasmic domain and the transmembrane domain can be seen clearly. Adding PIP_2 (red) causes the channel to occur in three slightly different conformations, marked with *, ** and *** (middle). CHS (green) leads to an even smaller spatial distance, as seen in the right picture. Taken from Kalyani Mathiharan et al., 2020.*[49]

6.9. Structure alignment

A structural alignment was performed to provide an overview of the differences between selected K_{IR} channels. The analysed channels with the corresponding PDB code, organism, resolution, root mean square deviation (RMSD) and primary literature are depicted in Table 5. First, single subunits of each channel as gz files were merged with the swiss PDB viewer by using biological assemblies.[50] Data was provided by the protein data bank. The generated PDB files were then aligned on a chicken $K_{IR}2.2$ channel co-crystallized with PIP_2 (PDB code 3SPI). The alignment is based on Cα atoms in the filter region and the aligned residues are given in the Table. Exceptions are 5KUK, 3SYA, 6C3O and 2WLL: the first three channels were aligned on 3SPI using an interactive magic fit with the swiss PDB viewer. This method enables an optimized structural alignment using a magic fit first and several cycles of improved fit afterwards, while the RMSD is held as low as possible. Using an interactive magic fit, the channels are aligned throughout the whole structure. 2WLL was aligned on its TMD1, which corresponds to the residues 79-103 in 2WLL and 60-84 in 3SPI. The RMSD of the whole channel is exceptionally high (19.51Å) in comparison to the other channels, which can be explained by an observed rotation of the cytoplasmic domain. All channels were visualized using PyMOL.[51] Residues of interest concerning the PIP_2 binding site as well as cholesterol binding sites were highlighted and their side chains were shown as sticks. Corresponding to the publications by Barbera et al. and Bukiya et al., different cholesterol binding sites were marked.[41,48] The results are presented in the following pages.

PDB code	**Organism**	**Channel**	**Resolution [in Å]**
3SPI	Chicken	Holo$K_{IR}2.2$	3.31
3SPJ	Chicken	Apo$K_{IR}2.2$ I223L	3.31
5KUK	Chicken	Apo$K_{IR}2.2$ K62W	2.00
3SYA	Mouse	Holo$K_{IR}3.2$	2.98
6C3O	Human	Apo$K_{IR}6.2$	3.90
2WLL	Burkholderia pseudomallei	K_{IR}Bac1.1	3.65
4LP8	Magnetospirillum magnetotacticum	K_{IR}Bac3.1	2.46
3ZRS	Magnetospirillum magnetotacticum	K_{IR}Bac3.1	3.05

PDB code	Aligned on	RMSD [in Å]	References
3SPI	T142-R149	set as standard	Hansen et al., 2011[52]
3SPJ	T142-R149	1.00	Hansen et al., 2011[52]
5KUK	Interactive magic fit	0.75	Lee et al., 2016[53]
3SYA	Interactive magic fit	1.27	Whorton, MacKinnon, 2011[54]
6C3O	Interactive magic fit	1.31	Lee et al., 2017[55]
2WLL	60-84 (3SPI) and 79-103 (2WLL)	19.51	Clarke et al., 2010[56]
4LP8	A95-L102	0.46	Zubcevic et al., 2014[57]
3ZRS	A95-L102	1.49	Bavro et al., 2012[58]

Table 5: The used K_{IR} channels with their corresponding PDB code, organism, resolution, the aligned sequences, RMSD and primary literature.

6.10. Structural insight in cholesterol binding sites

6.10.1. Eukaryotic K_{IR} channels, 3SPI

To give an overview of the putative cholesterol binding sites mentioned in this work, residues corresponding to the publication by Barbera et al. were marked in chicken $K_{IR}2.2$.[48] 3SPI was chosen because they had used it as well for their analysis. Here, the mentioned four possible cholesterol binding sites are separately depicted in Figure 22 to enable a better comparison. Residue numbers are only marked in one subunit. Binding site I looks similar to III and II is comparable with IV, as it has been described earlier.[48]
It can be seen that also lipophilic residues from the slide helix take part in the cholesterol binding process, like in binding sites III and IV. To be mentioned, C170 on TM2 seems to be orientated towards the inner cavity. However, this observed phenomenon may be dependent on the captured state of the crystal structure.

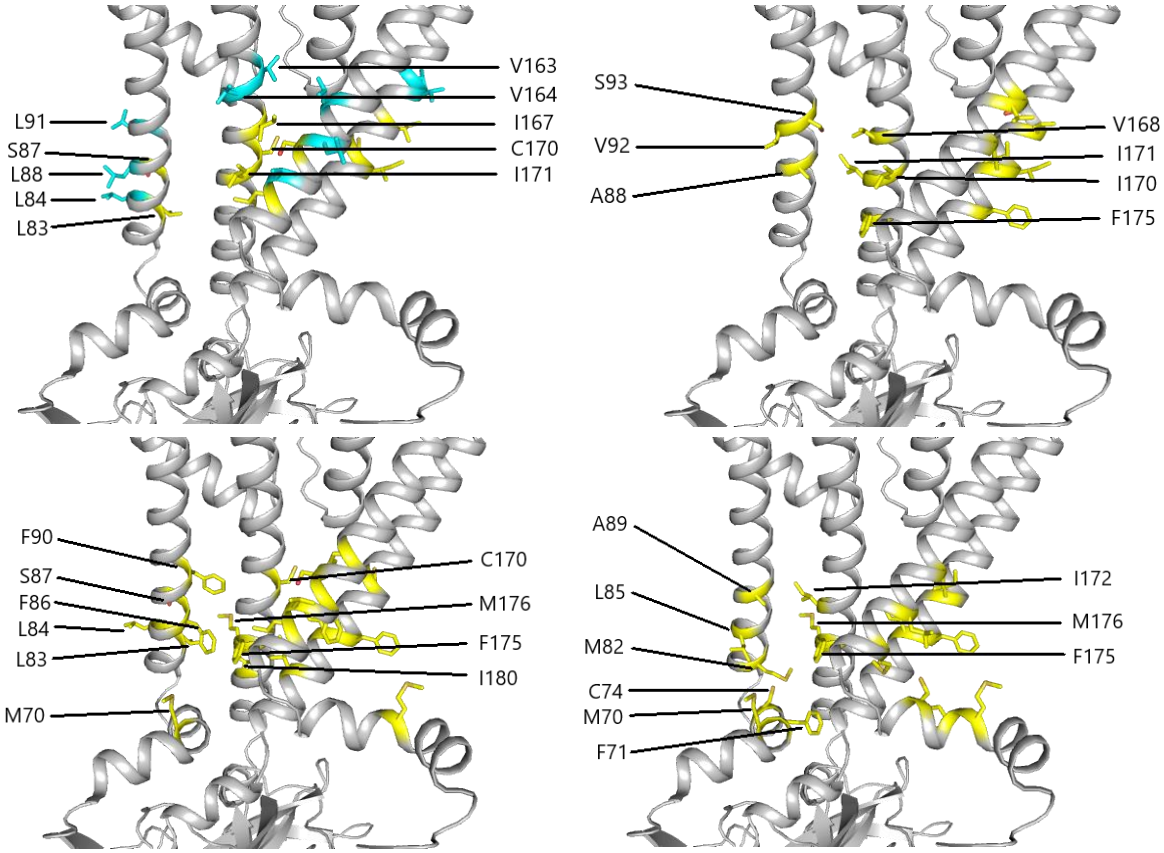


Figure 22: The first visualisation (top left) shows cholesterol binding site Ia (yellow residues) and Ib (light blue) in chicken $K_{IR}2.2$ (3SPI). Binding site II is depicted on the top right side, site III on the bottom left side and IV on the bottom right side. Two adjacent subunits are visualized in each picture, since the binding site lies within it. Carbons of the side chains of interest are coloured yellow with one exception: carbons concerning residues of binding site Ib are light blue to show the difference to Ia. Visualized with PyMOL.

6.10.2. 3SYA

Concerning $K_{IR}3.2$ (3SYA), the marked residues correlate with the two mentioned cholesterol binding sites by Bukiya et al.[41] As it can be seen in Figure 23, the cholesterol binding site contains primarily lipophilic residues. In both $K_{IR}2.2$ and $K_{IR}3.2$, lipophilic residues are facing towards the membrane. The predicted binding sites are similar. Most residues concerning the cholesterol binding site in $K_{IR}2.2$ and $K_{IR}3.2$ are located at the lower part of the transmembrane domains nearby the inner leaflet of the membrane bilayer. The binding of cholesterol molecules may lead to conformational changes and a rotation of the helices. However, experimental data are still missing since many publications present data generated from binding site predictions *in silico* only.

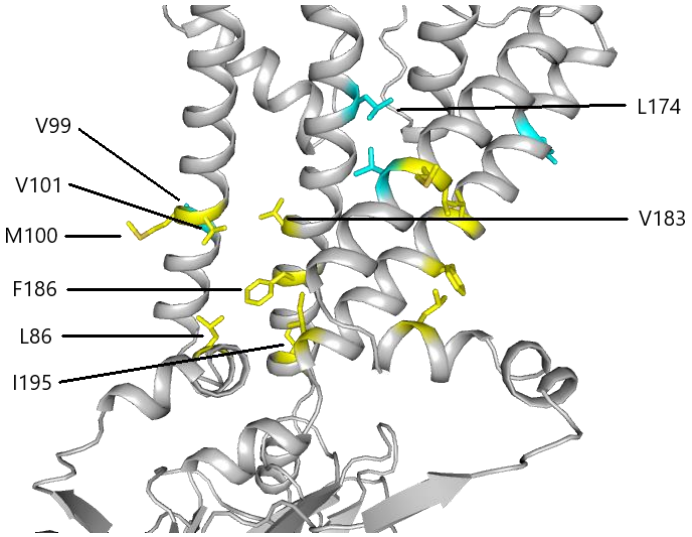


Figure 23: An overview of possible cholesterol binding sites in mouse$K_{IR}3.2$ (3SYA), relating to residues mentioned by Bukiya et al.[41] Residues concerning the first binding site are marked light blue, the ones from the second are yellow. Visualized with PyMOL.

6.10.3. Bacterial K_{IR} channels, 2WLL

Bacterial K_{IR} channels do not differ much structurally from eukaryotic K_{IR} channels concerning the cholesterol binding site. However, relating to a publication by Fürst et al., a special groove in K_{IR}Bac1.1, which does not occur in eukaryotic K_{IR} channels is marked in Figure 24 (see also chapter 6.3).[40] Important residues are F132, V133, S136 and G137. To sum up, K_{IR} channels in general are quite conserved among their transmembrane domains and their cholesterol binding site. In comparison to the PIP_2 binding site, no striking differences can be seen. TM2 is involved in the cholesterol binding process. However, one throwback in the interpretation of cholesterol binding sites is that cholesterol has not been co-crystallized with any K_{IR} channel yet and direct interactions like with PIP_2 remain hidden. Regarding bacterial K_{IR} channels, data is still lacking and further investigations concerning putative cholesterol binding sites need to be evolved as more residues could play important roles in the cholesterol binding process.

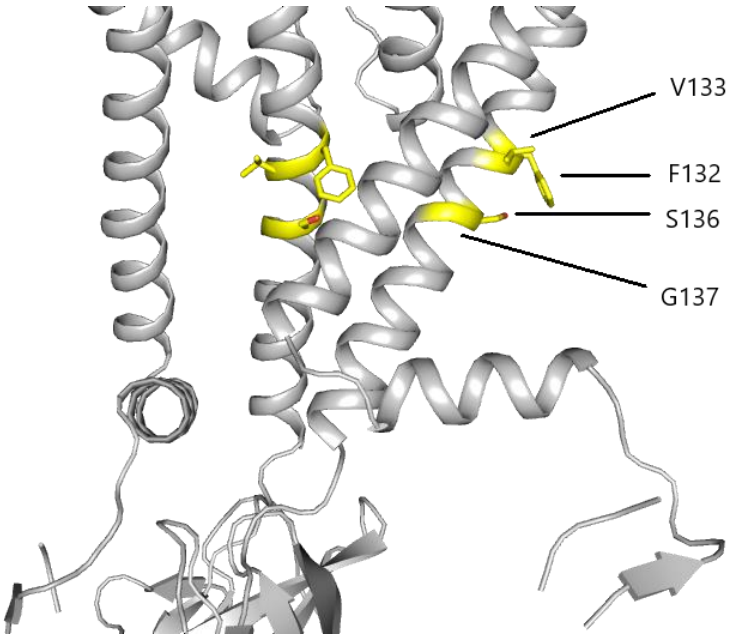


Figure 24: K_{IR}Bac1.1 (2WLL) and a groove of the cholesterol binding site. Visualized with PyMOL.

7. The role of PIP_2 in K_{IR} channels

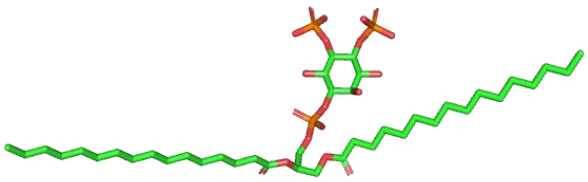


Figure 25: A possible structure of PIP_2 containing two saturated C-16 acyls. Visualized with PyMOL.

After discussing the inhibiting effect of cholesterol on K_{IR} channels, an activating factor (namely PIP_2, see Figure 25) needs to be presented in detail. In general, the interaction sites for PIP_2 are different to the ones of cholesterol. Every single K_{IR} channel requires PIP_2 for the opening process; and the PIP_2 sensitivity requires a secondary anionic phospholipid, which is described in detail later.[59] Not all K_{IR} channels are equally sensitive to PIP_2. For example, $K_{IR}1.1$ seems to bind PIPs in general more likely than members of the $K_{IR}3$ family.[60]

7.1. The effect of PI(P)s on K_{IR} channels

An overview of the effects of PIPs on different K_{IR} channels is given in Table 6.

Eukaryotic Channel	**Activation by**	**References**
$K_{IR}1.1$	$PI(4,5)P_2$ with PKA	Liou et al., 1999[61]
$K_{IR}2.1$	Primarily $PI(4,5)P_2$ Little by $PI(3,4,5)P_3$ $PI(3,4)P_2$ $PI(3,5)P_2$ PI(3)P	D'Avanzo et al., 2010 [62] D'Avanzo et al., 2013[63]
$K_{IR}3.1$	$PI(3,4)P_2$ $PI(4,5)P_2$ $PI(3,4,5)P_3$	Liu et al., 2019[64]
Prokaryotic Channel	**Inhibition by**	
$K_{IR}Bac1.1$ $K_{IR}Bac3.1$	$PI(4,5)P_2$	D'Avanzo et al., 2010[65] Linder et al., 2015[66]

Table 6: The effect of phosphatidylinositol phosphates on different K_{IR} channels. The most important difference is seen between eukaryotic and prokaryotic channels.

7.1.1. $K_{IR}1.1$

A study by Liou et al. revealed the PIP_2 dependency of ROMK1 channels and the role of protein kinase A (PKA).[61] ROMK channels have been found to be activated by $PI(4,5)P_2$, since they react with a rundown towards PIP_2 depletion. The channel activity can be experimentally restored by either adding PIP_2 or Mg-adenosine triphosphate (Mg-ATP), which promotes the generation of PIP_2 via phosphatidylinositol (PI) or phosphatidylinositol 4-phosphate (PI(4)P) in the cell by activating kinases. PKA is also presumed to be directly involved in $K_{IR}1.1$ channel phosphorylation, since several mutations in the PKA phosphorylation site are known to cause Bartter syndrome. The experiments of the study were performed on Xenopus laevis oocytes. Concerning the electrophysiological studies, on-cell configurations and afterwards inside-out patches were examined. PIP_2 dependency was shown by adding PIP_2 antibodies, which led to a channel inhibition. They demonstrated the specificity of the antibodies, as heat-inactivated antibodies and mouse IgG did not affect the channel activity. When PIP_2 was added excessively beforehand, the cells were prevented from a rundown caused by the antibodies. However, when PIP_2 was applied after the antibody-induced rundown, the channel activity could not be restored. If ROMK channels were activated by PKA and ATP, antibodies could not fully inhibit the channel activity, suggesting that the PIP_2 binding is strengthened by a channel phosphorylation. Concluding, it can be presumed that PKA is responsible for the ATP activation of ROMK channels by enhancing the interaction of PIP_2 with the channel. Further investigations with the ATP phosphorothiate derivate ATP[γS] showed that channel phosphorylation by PKA is insufficient for an activation. ATP[γS] is known to act as a PKA substrate like ATP, but kinases are not able to generate PIP_2 with it. Rundown channels could not be activated again by adding PKA together with ATP[γS]. However, the sensitivity to PIP_2 antibodies decreased, suggesting that channels were still phosphorylated. Experimental mutations in the PKA phosphorylation site in ROMK led to an increased PIP_2 antibody sensitivity. The PIP_2 binding site in $K_{IR}1.1$ is located carboxyterminal and contains phosphorylation sites that can be activated by PKA.[61]

7.1.2. $K_{IR}2.x$

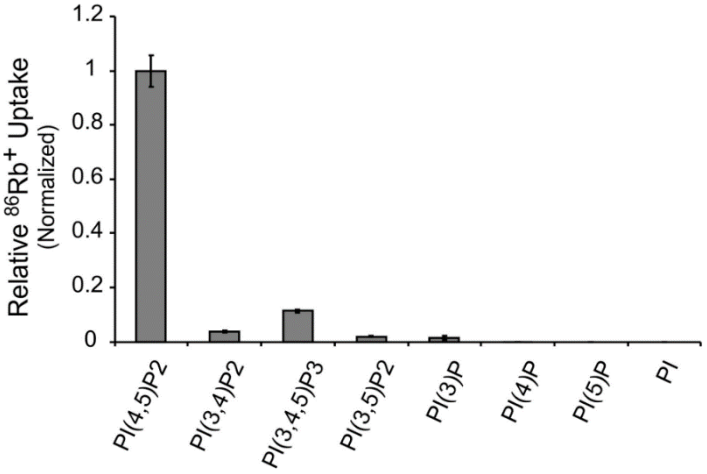


Figure 26: The open probability of $K_{IR}2.1$, measured by the relative $^{86}Rb^+$ uptake. $PI(4,5)P_2$ was set as standard. PI, PI(4)P and PI(5)P are not different to background signals. Taken from D'Avanzo et al., 2010.[69]

Phosphatidylinositol 4,5-bisphosphate seems to be the most important phospholipid for the opening process in K_{IR} channels. D'Avanzo et al. tested multiple variants of PIPs in $K_{IR}2.1$.[62] A standard of 100% was set by the channel activating property of $PI(4,5)P_2$, tests were measured as relative $^{86}Rb^+$ influx. The experiments were performed with liposomes containing 74% POPE (a phosphatidylethanolamine), 25% POPG (a phosphatidylglycerol) and 1% of each PI or PIP. In comparison to other PIPs, $PI(3,4,5)P_3$ caused the highest open probability in $K_{IR}2.1$ with 10% at a $PI(3,4,5)P_3$ concentration of 1%. Other PIPs like PI(3)P, $PI(3,4)P_2$ and $PI(3,5)P_2$ could activate the channel even less. However, PI, PI(4)P and PI(5)P showed no channel activation at all. The results of the experiment are presented in Figure 26. This finding supports the idea, that $PI(4,5)P_2$ is the major phospholipid concerning the activation process in K_{IR} channels; some PIPs are able to cause little channel activation at high concentrations as well.[62] Furthermore, the "headgroup" IP_3 alone is unable to activate any K_{IR} channels.[67]

7.1.3. $K_{IR}3.x$

Members of the $K_{IR}3$ family are different to $K_{IR}2.x$ channels in some ways because G proteins play an important role together with PIP_2 in the activation process. Furthermore, sodium ions play a supporting role.[68] The binding of PIP_2 causes the inner helix to rotate a bit but does not lead to a complete opening. It is seen controversy that Gβγ is absolutely required to bind to the cytoplasmic domain between two K_{IR} domains to generate completely open channels.[64] A study by Wang et al. sees Gβγ as essential factor for the

opening process.[69] They used planar lipid bilayers for their experiments and concluded, that both PIP_2 and Gβγ are absolutely needed for the opening process. They presume sodium ions only to support the mechanism. Figure 27 shows the activation process of GIRK channels via G proteins and PIP_2.

Here, the effect of the inhibiting neurotransmitter γ-aminobutyric acid (GABA) is presented schematically. G proteins consist of three different subunits (α, β and γ). The binding of GABA to the corresponding G protein-coupled receptor (GPCR) causes the heterotrimer to dissociate into $G\alpha_{i/o}$ and Gβγ, which further binds to GIRK channels.[69]

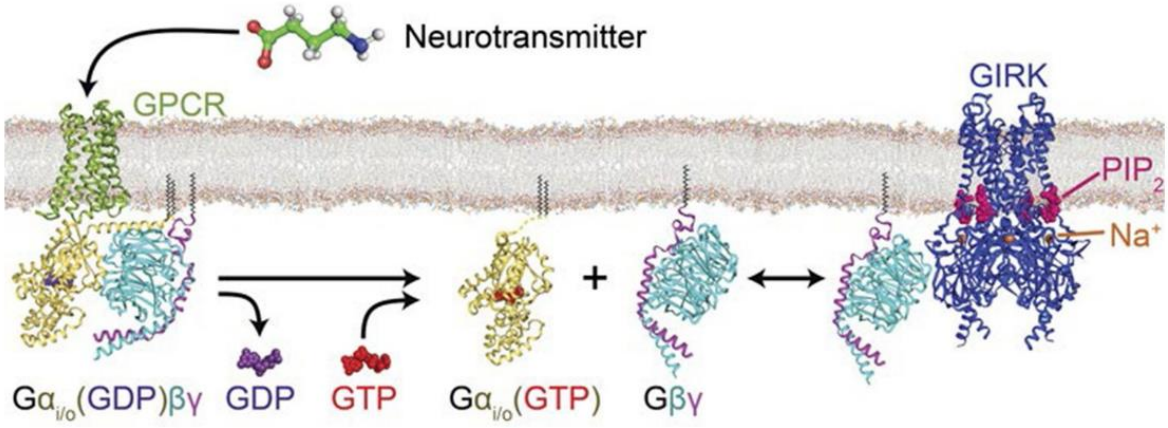


Figure 27: A visualisation of the activation process of $K_{IR}3$ channels in neuronal cell membranes. In this example, the inhibitory neurotransmitter γ-aminobutyric acid binds to a G protein-coupled receptor *(GPCR), which further causes an exchange of guanosine diphosphate (GDP) to guanosine triphosphate (GTP) and a release of the Gβγ subunit from the heterotrimer. Gβγ further binds to GIRK channels and causes them to open together with PIP_2 and sodium ions. Taken from Wang et al., 2016.*[71]

In contrast, a recent study by Bernsteiner et al. reveals new insights into the gating mechanism in $K_{IR}3.2$ channels.[68] PIP_2 is presumed to be sufficient to activate these channels while Gβγ is not essential. GIRK channels are known to show less PIP_2 sensitivity compared to $K_{IR}2$ channels and Gβγ is thought to strengthen the PIP_2 binding. It has been observed that PIP_2 leads to open mouse $K_{IR}3.2$ channels in a R201A mutated variant.[54] The mutation seems to mimic the Gβγ bound state as it causes a similar rotation in the cytoplasmic domain. Furthermore, MD simulations with PIP_2 but without Na^+ were performed in order to study gating mechanisms in $K_{IR}3.2$ channels. A possible explanation for the role of PIP_2 and Gβγ might be that Gβγ acts as an allosteric modulator, while PIP_2 is the main channel activator. X-ray structures reveal that PIP_2 is bound to GIRK2 in the

same way, independent on the presence of G proteins. Heterotetrameric $K_{IR}3.1/K_{IR}3.4$ channels are directly activated only by PIP_2, while Gβγ stabilizes the binding and therefore the open channel conformation.[68,70]

The roles of Gβγ and PIP_2 have been examined earlier in 1998 by Huang et al.[71] In detail, they expressed various K_{IR} channels ($K_{IR}1.1$, $K_{IR}2.1$, $K_{IR}3.1/K_{IR}3.4$ and $K_{IR}3.2$) in Xenopus laevis oocytes and performed electrophysiological studies. The experiments concerning GIRK channels shall be described in detail here. A rundown was induced by applying Mg^{2+} in order to deplete PIP_2. Afterwards, PIP_2 was added and the channel function could be restored. Applying Gβγ then did not activate the channels even more, as shown in Figure 28a. Furthermore, an addition of $G\alpha_i$ did not influence the recovery by PIP_2 as well (see Figure 28b). As presented in Figure 28c, Gβγ enables a faster channel activation by PIP_2 in comparison to PIP_2 alone. In another experiment, channels were not allowed to run down entirely and Gβγ was added afterwards. PIP_2 was unable to activate the channels more once the maximum was reached by Gβγ (see Figure 28d). An overexpression of Gβγ in $K_{IR}3.1/K_{IR}3.4$ as well as in $K_{IR}3.2$ caused the channels to be less sensitive to PIP_2 antibodies, as shown in Figure 28e and f. To sum up, the study sees PIP_2 to be sufficient for GIRK channel activation, but Gβγ strengthens the PIP_2 binding.[71]

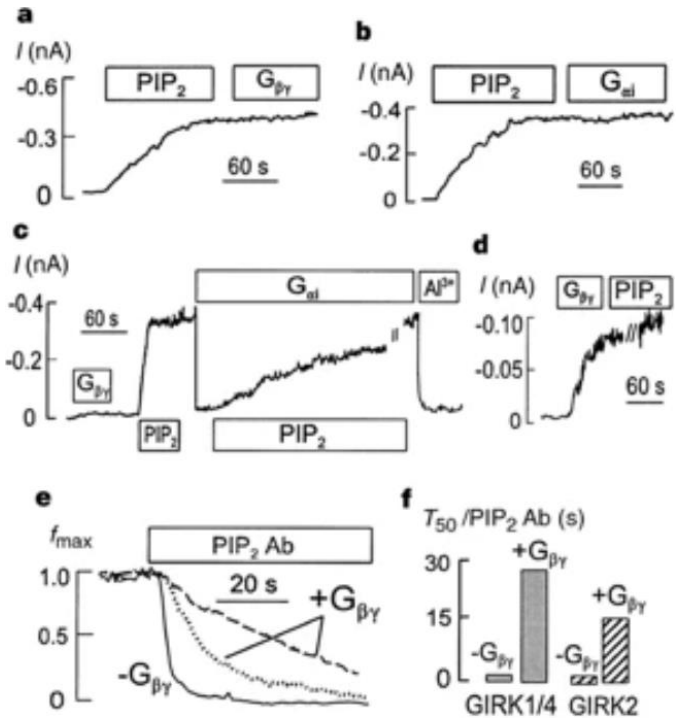


Figure 28: Correlations of Gβγ and PIP_2 in $K_{IR}3.1/K_{IR}3.4$ (a-f) and $K_{IR}3.2$ (f). Gβγ (a) and Gαi (b) do not influence the maximized channel activity after a rundown. Gβγ accelerates the recovery time by PIP_2, Al^{3+} inhibits channel activity (c). The examined channels could not be activated further by PIP_2, when a maximal activity was reached by Gβγ (d). The sensitivity towards PIP_2 antibodies (Ab) decreases in the presence of Gβγ (e). Gβγ causes the time for half-maximal channel inhibition (T50) by PIP_2 antibodies to increase (f). Thus, Gβγ is responsible for a minor PIP_2 antibody sensitivity. Taken from Huang et al., 1998.[62]

A difference in the PIP_2 binding lies within a single lysine at position 64 in mouse $K_{IR}3.2$, which interacts with the 4' phosphate of PIP_2 via a hydrogen bond. An equivalent position with this property has not been observed in $K_{IR}2.2$. The corresponding residue in human $K_{IR}3.2$ is K62. $K_{IR}3.4$ and its co-assembly with $K_{IR}3.1$ can be activated by $PI(3,4,5)P_3$ and $PI(4,5)P_2$ enormously with almost the same potency. Less activation is provided by $PI(3,4)P_2$ and $PI(3,5)P_2$.[60] However, $K_{IR}3.2$ has different PIP affinities than the previously mentioned channels. A study by Liu et al. used native ion mobility mass spectrometry (MS) to investigate the affinity of mouse $K_{IR}3.2$ towards different types of phospholipids.[64] The experiments were performed with native $K_{IR}3.2$ and its mutation R201A. The mutation enables studies of PIP binding and its effects on the orientation of the cytoplasmic domain. Wild type $K_{IR}3$ channels without G proteins show only weak PIP binding activity and the isolation of $K_{IR}3.2$ together with Gβγ has not been experimentally successful. The tested phospholipids were PI(4)P, $PI(3,4)P_2$, $PI(3,4,5)P_3$ and $PI(4,5)P_2$ containing two oleoyl chains (18:1-18:1), two dioctanoyl chains (8:0-8:0) or one stearoyl chain (18:0) in combination with one arachidonoyl chain (20:4). An important finding of this study is that the affinity of $PI(3,4)P_2$ to the wild type $K_{IR}3.2$ channel is similar to the affinity of $PI(4,5)P_2$. The length and saturation grade of the acyl chains do not play important roles. The affinity among different $PI(4,5)P_2$ with altered acyl chains was similar in the R201A mutant as well. On the other hand, the mutation resulted in a loss in the binding affinity of $PI(3,4)P_2$. In comparison to the wild type, the mutant showed an altered selectivity to PIPs: it is more affine to $PI(4,5)P_2$ and less to all the other isoforms.[64]

A more recent study was performed by Qiao et al. with a similar experimental method using the same PIPs in $K_{IR}3.2$ as described above.[60] It was reported that PI(4)P showed low binding affinity, suggesting that the 5' phosphate is essential for PIPs to bind successfully. To gain insight into the importance of K64 in $K_{IR}3.2$, a K64Q mutant was introduced. Glutamine has been chosen because it occurs at the equivalent position (51) in $K_{IR}2.2$. Interestingly, the mutation led to an altered sensitivity of the channel towards the acyl chains of the PIP molecules like it has been observed in $K_{IR}3.1/K_{IR}3.4$. Another mutation (R92P, corresponding to R90 in human $K_{IR}3.2$) was introduced because arginine at equivalent positions is widely conserved among most K_{IR} channels, with the exception of $K_{IR}6.2$, which has proline instead. R92 in $K_{IR}3.2$ is located in the second transmembrane

helix and proline is known to introduce a rigid kink. The R92P mutation resulted in a promiscuous PIP binding with a surprising increase in the PI(4)P affinity in comparison to the wild type. To examine the importance of a residue concerning the binding of the 5' phosphate, K194 in $K_{IR}3.2$ (corresponding to K192 in human $K_{IR}3.2$) was mutated to alanine. Most PIPs were still able to bind to the K194A mutant, but small decreases in the affinity to PIPs containing a 5' phosphate, were observed. $PI(3,4,5)P_3$ had the highest affinity to the channel.[60]

7.2. Electrophysiological studies on K_{IR} with different PIP_2 concentrations

D'Avanzo et al. performed electrophysiological studies to provide evidence for the essential function of PIP_2 in K_{IR} channels.[62] They used saccharomyces cerevisiae as expression system for $K_{IR}2.1$ and $K_{IR}2.2$ channels. In one experiment, the flow of $^{86}Rb^+$ was measured in liposomes with the previously separated K_{IR} proteins. The used liposomes always had a defined composition of POPE and POPG in a 3:1 ratio. 0.01% to 3% PIP_2 was then applied while altering the POPE concentration. The uptake of $^{86}Rb^+$ increased at higher PIP_2 concentrations while a

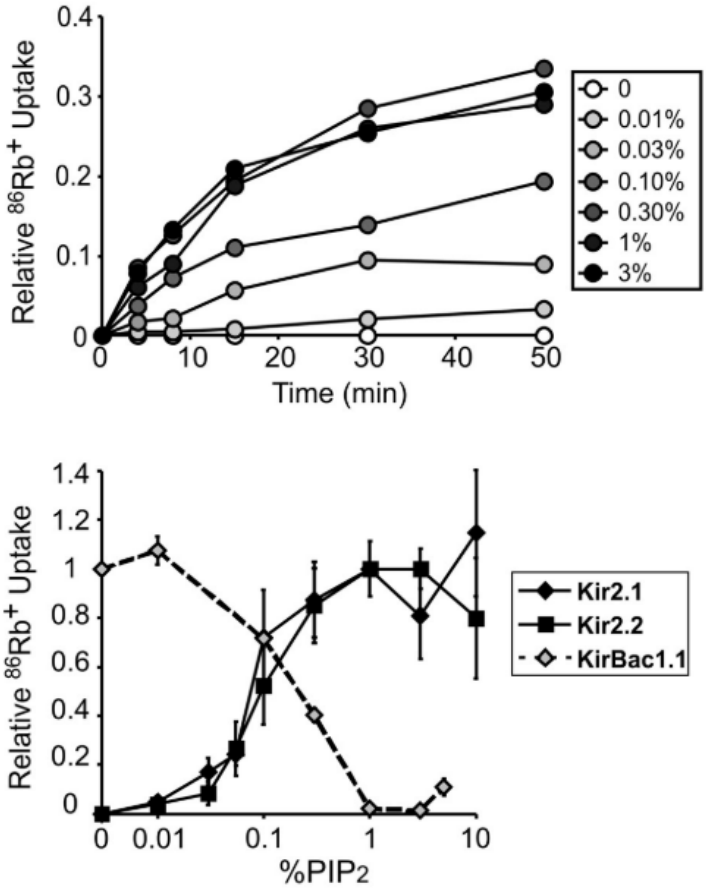


Figure 29: The uptake of $^{86}Rb^+$ depending on different PIP_2 concentrations. The upper graph shows the relative influx in $K_{IR}2.1$ over time. The second graph depicts the different reactions towards PIP_2 concentrations in $K_{IR}2.1$, $K_{IR}2.2$ and $K_{IR}Bac1.1$. Taken from D'Avanzo et al., 2010.[65]

plateau was reached at about 1% PIP_2. It has also been shown how bacterial K_{IR} channels (K_{IR}Bac1.1) react to different PIP_2 concentrations in relation to eukaryotic channels. PIP_2 has the opposite effect on eukaryotic and prokaryotic K_{IR} channels and the observed human K_{IR} channels seem to be more sensitive to PIP_2 than bacterial K_{IR}. The results of both experiments are presented in Figure 29.[62]

7.3. PIP_2 in bacterial K_{IR} channels

While PIP_2 is an important activating factor in eukaryotic K_{IR} channels, it inhibits the prokaryotic channel K_{IR}Bac1.1. This may be caused by a lack of key residues in K_{IR}Bac1.1, which occur in eukaryotic K_{IR} channels and act as two short linker regions between the cytoplasmic and the transmembrane pore-forming domain. The mentioned linker region in K_{IR}Bac1.1 is three amino acids shorter. This difference seems to be the reason for the important spatial distance between the two domains in eukaryotic K_{IR}. As a result of the proximity in K_{IR}Bac1.1, channels are more likely to be open because of interactions between the cytoplasmic domain and the slide helix. PIP_2 is not only unnecessary for this process, but even inhibiting due to a destabilisation. The closed state is favoured because PIP_2 separates the two mentioned parts. It must be noticed that PIP_2 (or phosphoinositide lipids in general) usually do not occur in bacterial membranes. Instead, the major lipids are PE and phosphatidylglycerol (PG). If bacteria contained PIP_2 in concentrations that are usual for mammalian cells, K_{IR}Bac1.1 would not be functional at all and vice versa: without PIP_2, the activity of many eukaryotic K_{IR} channels is decreased. The introduction of the additional short amino acid sequence in eukaryotic organisms is thought to be an adaption to the evolutional changes in the composition of the plasma membrane. A sequence alignment showing the mentioned differences between eukaryotic and prokaryotic K_{IR} channels can be seen in Figure 30.[65]

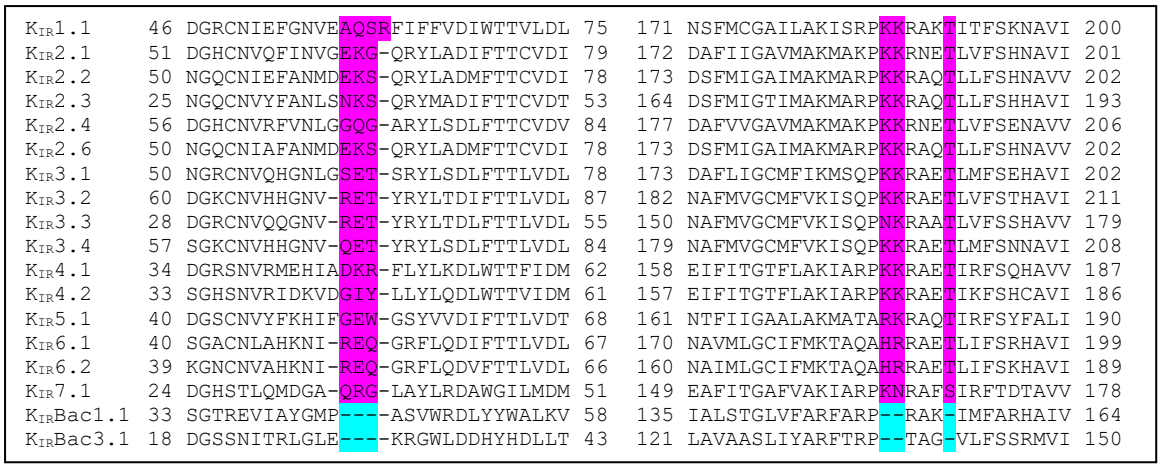


Figure 30: Sequence alignment of different eukaryotic K_{IR} channels from 1-7 as well as K_{IR}Bac1.1 and K_{IR}Bac3.1. The difference that leads to an altered PIP_2 reaction to human K_{IR} (pink) is depicted in blue.[72]

Eukaryotic and prokaryotic K_{IR} channels in general do not share many amino acid sequences. Lindner et al. evaluated the gating process in bacterial K_{IR} channels, which shall be discussed here as well.[66] MD simulations were performed with native K_{IR}Bac1.1. In general, conformational changes in the cytoplasmic domain and the transmembrane domains are required for the gating process. Especially a part of the TM2 is responsible for a constriction in the closed state, namely the HBC gate. X-ray structures of an open channel conformation of K_{IR}Bac3.1 have been revealed by introducing a gain of function mutation (S129R).[66] With K_{IR}Bac1.1 as an example, generating X-ray structures from a native channel is actually impossible, since the activation process in bacterial K_{IR} channels requires anionic lipids. Unfortunately, they cannot be co-crystallized with the channel for the measurement.[72] A similar mutation (G143E) in K_{IR}Bac1.1, which is located between two TM2 subunits, was used for MD simulations. In the study, the deprotonated G143E mutation caused a conformational change in the cytoplasmic domain and the HBC gate, leading to an open channel conformation. Interestingly, trying to express the mentioned mutant in E. coli did not work well, as only a few colonies could be obtained. This finding seems to be consistent with the fact that G143E mutated channels are highly active and therefore even toxic to the E. Coli strain. Furthermore, the study provides evidence for the pH dependency of the mutated channel, since only the deprotonated form showed the required conformational changes.[66]

Another study by Amani revealed how bacterial K_{IR} channels react towards different lipids and also PIP_2.[72] Figure 31 shows a model of K_{IR}Bac1.1. An important result of the study is that K_{IR}Bac1.1 is conductive in lipid bilayers containing POPC and POPG in a 3:2 ratio. To prove that the channel activity is above a basal activity, PIP_2 was added in various concentrations from 1.25 to 5%. At 3% PIP_2, channels were almost completely inhibited. Furthermore, lipid bilayers consisting of zwitterionic POPC cause K_{IR}Bac1.1 to be inactive. On the other hand, the channel proteins seem to strengthen the bilayer, since it is leaky in experiments without K_{IR}Bac1.1.[72]

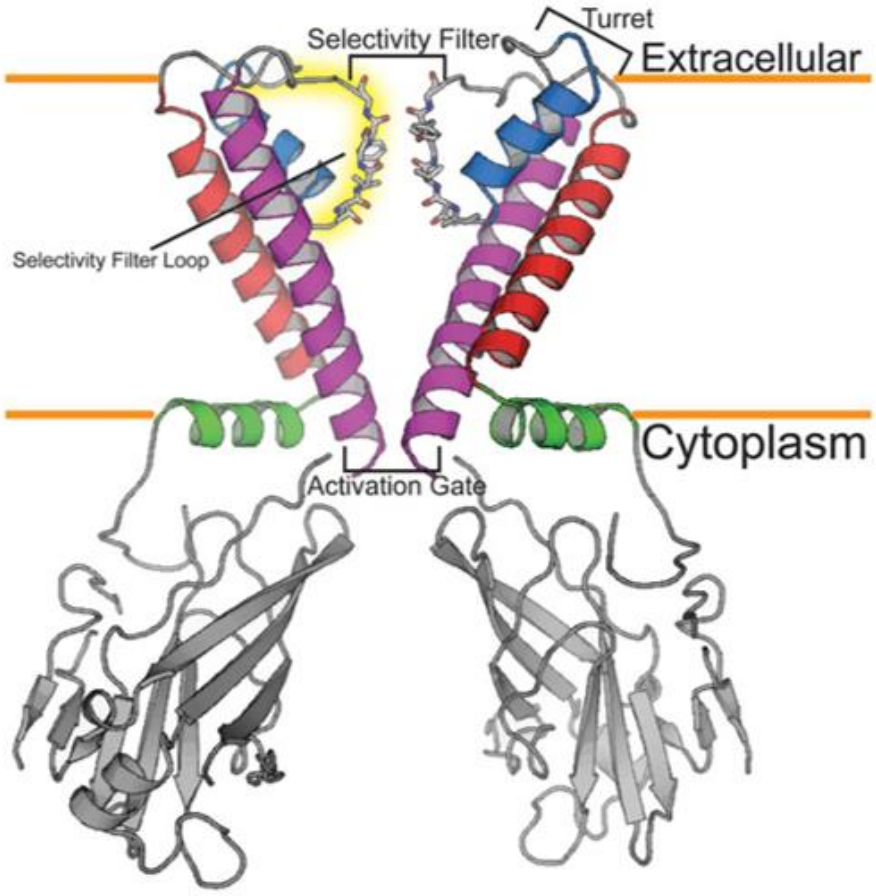


Figure 31: A model of K_{IR}Bac1.1. TM1 is depicted in red, TM2 in purple, the slide helix in green and the pore helix in blue. The cytoplasmic part of the channel is held in grey. It can be seen clearly that the activation gate is located on the edge of the inner plasma membrane. Taken from Amani et al., 2020.[52]

7.4. Primary PIP_2 binding site

Each subunit of K_{IR} channels enables the binding of one PIP_2 molecule. The primary binding site is located between the transmembrane and cytoplasmic domain. An interaction with PIP_2 results in an enormous (6 Å) conformational change of the cytoplasmic towards the TM domain, which is visualized in Figure 33. In detail, the inositol group of PIP_2 interacts with the cytoplasmic side, whereas the acyl chains stay in the lipophilic environment of the K_{IR} helices.[52] The length and saturation grade of the fatty acyl chains generally do not affect the affinity to the binding site at all, like in $K_{IR}2.1$.[60,73] On the other hand, the heterotetrameric channel $K_{IR}3.1/K_{IR}3.4$ prefers 18:0-20:4 fatty acyl chains of PIP_2. Another member of the same family, $K_{IR}3.2$, does not show preferences in the length and saturation grade of the lipid tails.[60,64] A sequence of Arg-Trp-Arg at the positions 79-81 ($K_{IR}2.2$) has been found to be the target of the 1' phosphate of the inositol group. The sequence is widely conserved among other K_{IR} channels as well, while the first amino acid can be Arg, Lys or Gln. A sequence alignment containing this motif is presented in Figure 32. The binding site is located at the edge to the TM domain. Tryptophan seems to play other important roles as well: it interacts with an acyl chain and acts as an anchor for the outer helix at the membrane. The glycerol backbone of PIP_2 is associated with the transmembrane domain. Concerning the inositol group, the phosphates at position 4' and 5' are known to interact with mainly positively charged amino acid residues: three lysines at the positions 183, 188 and 189 and one arginine at position 186 ($K_{IR}2.2$). The specific recognition of PIP_2 is managed by the cytoplasmic region as any other lipids can also bind to the TM domain. The binding of PIP_2 induces the structure of an interfacial helix and a tether helix

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KIR1.1  66 VDIWTTVLDLKWRYKMTIFITAF 88
KIR2.1  70 ADIFTTCVDIRWRWMLVIFCLAF 92
KIR2.2  69 ADMFTTCVDIRWRYMLLIFSLAF 91
KIR2.3  44 ADIFTTCVDTRWRYMLMIFSAAF 66
KIR2.4  75 SDLFTTCVDVRWRWMCLLFSCSF 97
KIR2.6  69 ADMFTTCVDIRWRYMLLIFSLAF 91
KIR3.1  69 SDLFTTLVDLKWRWNLFIFILTY 91
KIR3.2  78 TDIFTTLVDLKWRFNLLIFVMVY 100
KIR3.3  46 TDLFTTLVDLQWRLSLLFFVLAY 68
KIR3.4  75 SDLFTTLVDLKWRFNLLVFTMVY 97
KIR4.1  52 KDLWTTFIDMQWRYKLLLFSATF 74
KIR4.2  51 QDLWTTVIDMKWRYKLTLFAATF 73
KIR5.1  59 VDIFTTLVDTKWRHMFVIFSLSY 81
KIR6.1  58 QDIFTTLVDLKWRHTLVIFTMSF 80
KIR6.2  57 QDVFTTLVDLKWPHTLLIFTMSF 79
KIR7.1  42 RDAWGILMDMRWRWMMLVFSASF 64
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Figure 32: A sequence alignment of the 1' phosphate binding site (blue). The first amino acid can be Lys, Gln or Arg. $K_{IR}6.2$ shows a difference at the third position of the motif containing proline instead of arginine, which is marked pink.

containing K188 and K189 ($K_{IR}2.2$). A network of hydrogen bonds is responsible for the interaction of cytoplasmic regions with the tether helix. The binding site contains a head group, which results from the conformational changes. A lipid without the important inositol group would not lead to these changes; only the binding to the transmembrane domain would still be possible. For example, short fatty acyls attached to pyrophosphatidic acid (PAA) can interact with the PIP_2 binding site but do not lead to the important conformational change of the cytoplasmic towards the transmembrane domain. PAA only contains phosphoric acid and no inositol ring, which seems to be essential.[52] PAA does not show any synergistic effect to an activation by PIP_2 but acts as competitive inhibitor. Dependent on PPA concentrations, the channel activity is decreased to totally inhibited.[59] To sum up, other molecules than PIP_2 cannot act as channel opening modulators. PIP_2 interacting with its binding site leads to an opening of the inner helix gates and initiates the opening of the channel pore. Another constriction is the G-loop, which can be found at the peak of the cytoplasmic domain. It is thought to act as a gate and its conformation depends on PIP_2 binding. Nevertheless, the latter binding does not influence the G-loop gate much because it is open in both states of the ion channel.[52]

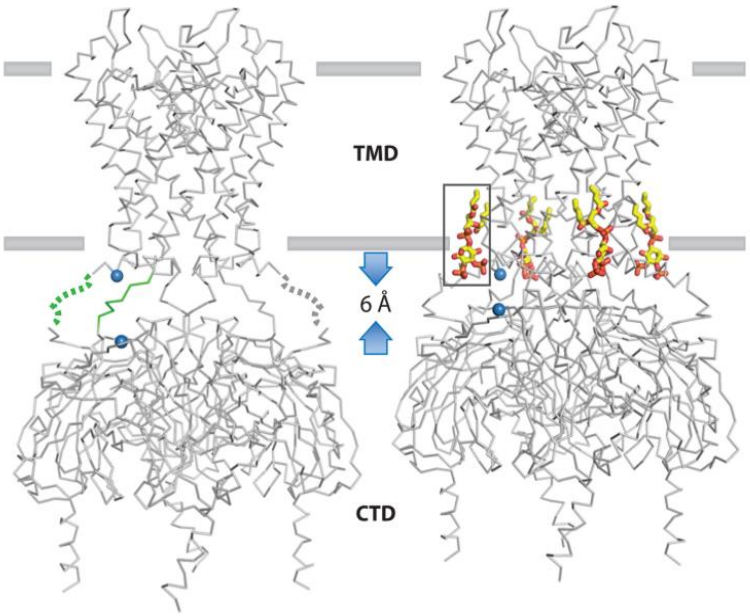

Figure 33: A visualisation of apo $K_{IR}2.2$ (left) and its conformational changes caused by PIP_2 binding (right). The carbons of PIP_2 are depicted in yellow, phosphor in orange and oxygen in red. The blue dots show random amino acid residues for a better view of the distance changes. Taken from Hansen et al., 2011.[52]

7.5. Secondary anionic phospholipids

Many different lipids can act as agonists at the secondary anionic phospholipid binding site, for example phosphatidic acid (PA), PS, CL, PI or PG.[59] It has to be mentioned that high concentrations of anionic phospholipids, but especially PIPs lead to a competitive inhibition of the K_{IR} channel activity, regarding the PIP_2 dependency. This has been shown in a $K_{IR}2.1$ model and leads to the possible conclusion that especially PIPs can interact with PIP_2 binding sites but are unable to activate the channel.[73] Anionic phospholipids in general make up over 15% of all kinds of lipids and about 0.4% to 1.5% of anionic phospholipids is PIP_2.[53,59]

An increase of the first mentioned lipids in the membrane leads to an enhanced open probability and unitary conductance. Anionic phospholipids in general have been found to bind at two sites: the PIP_2 binding site, which has been described before, and a more distinct second site, that does not provide specific interactions; the efficacy of many kinds of lipids is similar. Generally, all lipids are more affine to the first site. In case of an occupation by PIP_2, anionic phospholipids tend to bind at the second site as well. It is also possible that two different PIP_2 molecules interact with both sites at once. On the other hand, if the second binding site is occupied by PIP_2, all the anionic phospholipids bind to the first site. The PIP_2 binding to its first site leads to an increase of efficacy concerning

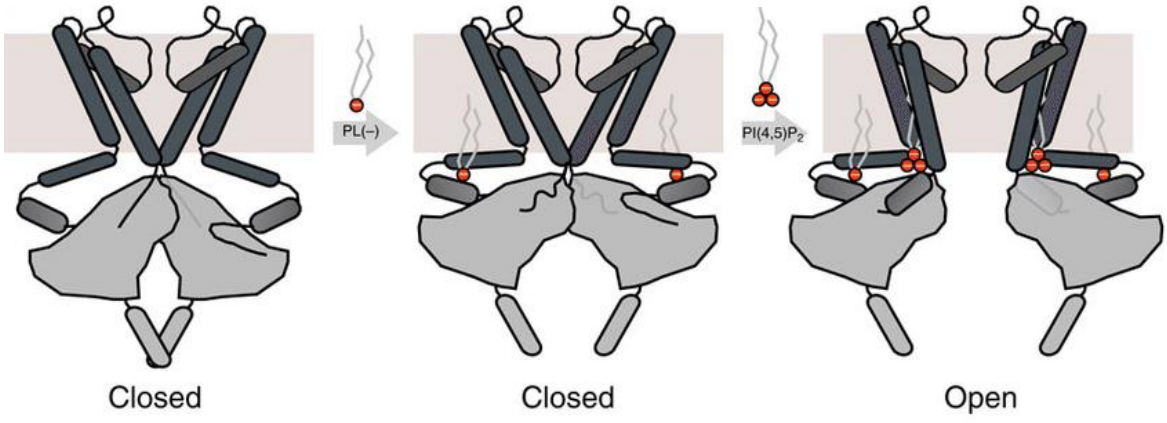

Figure 34: The visualisation of a K_{IR} channel in the closed state does not contain any molecule binding to it in the left picture. The binding of a secondary anionic phospholipid (middle) leads to a conformational shift of the cytoplasmic domain towards the transmembrane domain, enabling PIP_2 molecules to bind to their primary binding site (right), which further results in an opening of the channel pore. Taken from Lee et al., 2013.[59]

anionic phospholipids interacting with the secondary site and vice versa. A visualisation of the opening process by PIP_2 and a secondary anionic phospholipid is presented in Figure 34.[59]

In $K_{IR}2.1$, two important lysine residues at position 64 and 219 have been discovered, which are responsible for the binding of a secondary anionic phospholipid. The positive charge of lysine can interact with the negatively charged phospholipids. Disulphide bonds between K64C and K219C are probable when both mutations are expressed simultaneously, while the cysteine in a single mutation (K64C or K219C) is more likely to be reduced. Evidence for this finding has been provided by changing the two lysines to cysteine and performing electrophysiological studies. Reduced channel activity was observed, the double mutants show almost no channel activity. At first, cell-attached configurations were measured. After an excision, inside-out currents were analysed, and later 10µM of the polyamine spermine was added. The off-cell patches without spermine showed higher currents in general, which can be explained by a weaker rectification process due to fewer polyamines, when the patch is recessed. The analysed channels were wild type $K_{IR}2.1$, a cysteine-less mutant with numerous varied cysteine residues except for the cysteines on positions 122 and 154, a K64C mutant, a K219C mutant and a double mutant (K64C and K219C). While the cysteine less channels are comparable with the wild type, a reduction in the channel activity can be seen in all the other mutants. The results are presented in Figure 35.[59]

Artificially adding a lipophilic acyl containing ten carbons to a K64C mutation and therefore tethering it to the membrane leads to a higher affinity for PIP_2, almost independent of secondary anionic phospholipids. The same decyl-modification of K219C still showed a dependency on secondary anionic phospholipids. Double mutants of K64C and K219C with lipophilic modifications provide little more channel activity than without adjustments, what may be due to the absence of disulphide bonds among each other. These findings suggest that K64 is the major residue of the secondary anionic phospholipid binding site.[59]

Another study by Lee et al. examined the effect of another mutation at K62 (chicken $K_{IR}2.2$, corresponding position of K64 in human $K_{Ir}2.1$) by altering lysine to tryptophan.[53] This change results in a higher PIP_2 sensitivity, independent of secondary

anionic phospholipids. As expected, wild type K_{IR} channels did not show any channel activity in absence of POPG; in contrast, K64W channels were quite active and adding POPG did not lead to a major increase anymore. Wild type channels still needed POPG for their PIP_2 sensitivity. Concerning other channels than the $K_{IR}2$ family, the mentioned residues, which are responsible for the tethering to the membrane, are not highly conserved. This finding suggests that other ligands than secondary anionic phospholipids might also lead to a membrane tethering.[53]

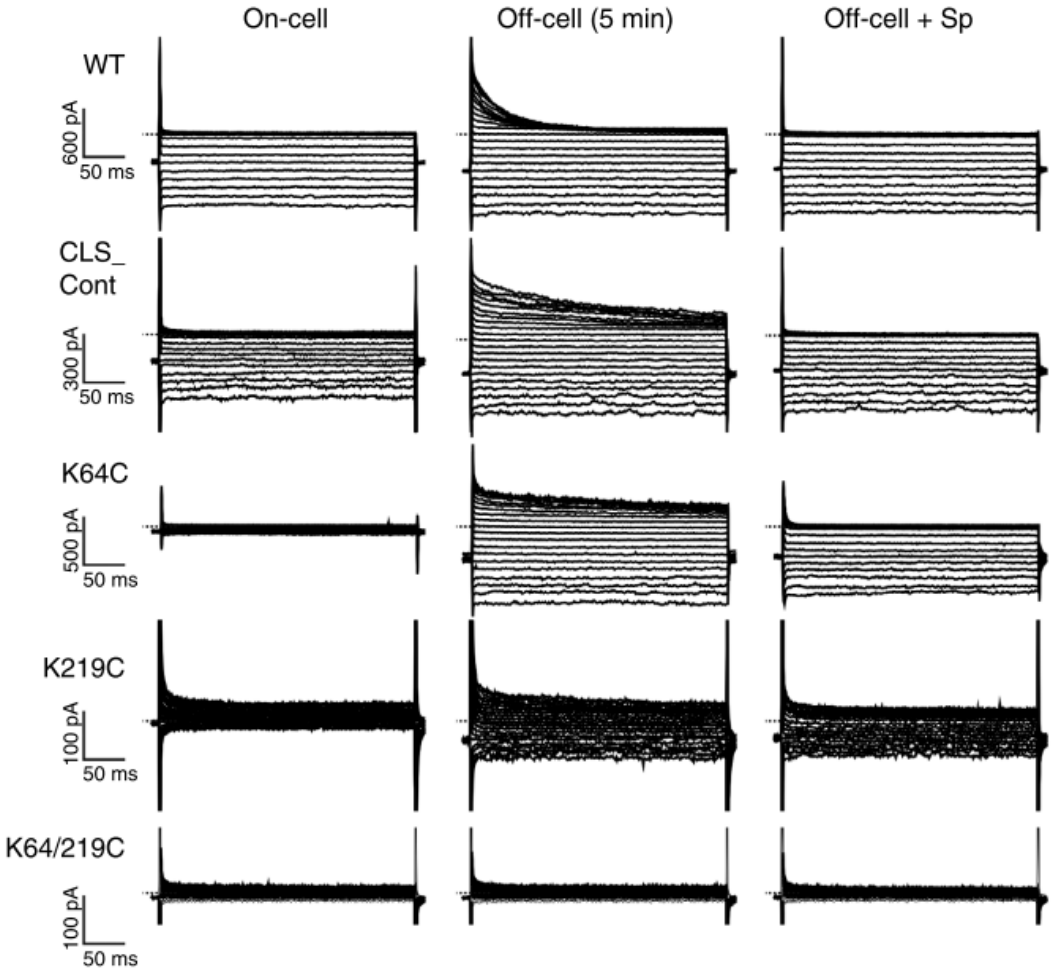

Figure 35: Electrophysiological studies showing $K_{IR}2.1$ channel activity. The analysed channels are wild type $K_{IR}2.1$ (WT), a cysteine less mutant (CLS_Cont) and the mutations K64C, K219C and K64C/K219C. The recordings on the left were observed on-cell, the pictures in the middle show inside-out patches after five minutes and the graphs on the right depict the recordings after adding 10µM spermine (Sp). Taken from Lee et al., 2013.[76]

7.5.1. Importance of heterogeneous lipid compositions

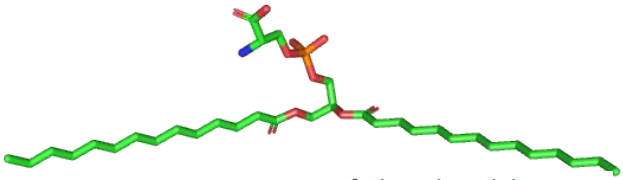

Figure 36: A representative of phosphatidylserine. Visualized with PyMOL.

A study by Duncan et al. shows the importance of different lipid species in K_{IR} channels.[74] Therefore, they performed a CG molecular dynamics simulation of chicken $K_{IR}2.2$, which is known to have no glycosylation sites, within a lipid bilayer consisting of the following lipids at the inner leaflet: PC, PE, cholesterol (Chol), PS (see Figure 36) and PIP_2 as well as PC, PE, Chol, Sph and a ganglioside (namely GM3, a subgroup of sphingolipids) at the outer leaflet.[29,74] It has been observed that PIP_2 has the best affinity to $K_{IR}2.2$ concerning the inner leaflet, followed by PS, which shows the nearest radial distribution to the channels in simulations without PIP_2. Both distributions are independent of cholesterol presence. GM3 seems to be the leading lipid interacting with $K_{IR}2.2$ at unspecific sites at the outer leaflet. Its glycan group interacts relatively further away from the centre of the plasma membrane than the headgroups of Sph and PC. The latter lipids are closer to the extracellular mouth of $K_{IR}2.2$. Neither the interactions of PIP_2 nor of cholesterol are influenced by GM3, which may be caused by its localisation in the outer leaflet. The headgroups of PIP_2 and PS interact with $K_{IR}2.2$ at TM and cytoplasmic domains.[74] The interaction of numerous cholesterol molecules is basically more dispersed, located near the centre of the plasma membrane and lower than the contacts of PIP_2, PS and GM3. Especially in absence of PIP_2, PS can also interact with the typical PIP_2 binding sites, including K62 and K220 in chicken $K_{IR}2.2$(K63 and K220 in human $K_{IR}2.2$). PIP_2 has been found to interact with its typical primary binding site (which has been described before) as well as a possible second binding site, but it also shows numerous unspecific interactions. However, the interaction with K220 is weaker than with K62. K220C mutations result in a greater maximal channel activity in comparison to K62C, confirming the importance of K62. The longest residence time at the primary PIP_2 binding site shows PIP_2 itself, followed by PS. The latter lipid has a lower affinity due to its smaller head group. It has been shown that PIP_2 does not have high affinities to the secondary anionic phospholipid binding site. Interestingly, some PS molecules have been found to be

trapped behind PIP_2 at the primary site in the simulation. This may give an explanation to longer residence times of PS while PIP_2 is present than while it is absent. However, the residence time of PIP_2 does not show any changes. PS is presumed to interact with the same sites as PIP_2 does but is outcompeted when PIP_2 is present as well. Another important finding is that the interaction of PIP_2 with its primary binding site does not depend much on PS but vice versa, the residence time of PS binding to the secondary site increases in the presence of PIP_2 binding to the primary site. A probable explanation for this mechanism is the altered spatial difference between R78 (positively charged) and K62 during the interaction of PIP_2 at the primary binding site. In absence of PIP_2, the two mentioned residues are closer together. As a result, PS can be found rather in between the two residues, the residence time at K62 decreases and PS binds favourably to R78 or R80.[74]

7.5.2. $K_{IR}2$ in liposomes with various lipid compositions

To gain further insight into the role of the lipid composition of the plasma membrane, a study by Cheng et al. used liposomes consisting of defined lipid concentrations.[75] Therefore, $K_{IR}2.1$ and $K_{IR}2.2$ channels were previously expressed in Saccharomyces cerevisiae and embedded into liposomes afterwards. The liposomes composed of 75% POPE together with 25% POPG. To examine the channel activity, $^{86}Rb^+$ flux was measured. An important finding is that the channel activation via PIP_2 is also dependent on POPG, as seen in Figure 37. The absence of POPG in liposomes consisting of POPE only shifts the sensitivity of PIP_2. Thus, higher PIP_2 concentrations are necessary to

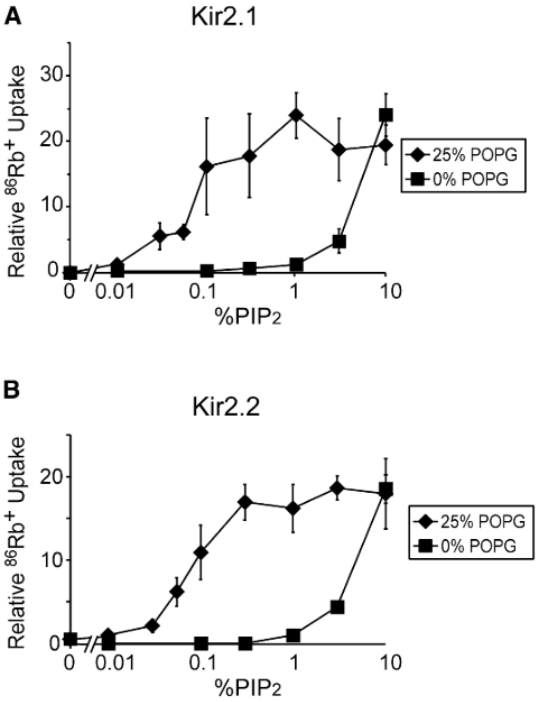

Figure 37: The flux of $^{86}Rb^+$ representing channel activity in $K_{IR}2.1$ (A) and $K_{IR}2.2$ (B). In the absence of POPG, significant higher (non physiological) PIP_2 concentrations are needed for channel activation. Taken from Cheng et al., 2011.[75]

provide channel activity. However, the presence of POPG leads to an increased unitary conductance and an enhanced open probability as well. To assess whether specifically POPG is responsible for the increased PIP_2 sensitivity, other similar lipids with differences in their headgroup were tested in liposomes containing $K_{IR}2.1$, results are presented in Figure 38. The used lipids in contrast to POPG were POPS (a phosphatidylserine), POPC, EPC (a phosphatidylcholine), oleoyl CoA, POPA (a phosphatidic acid), PI, CL and DGS-NTA (a lipid containing an iminodiacetic acid headgroup). The liposomes also contained 1% PIP_2. Liposomes could not be generated successfully with 3%, 10% and 30% EPC. The different kinds of lipids seem to be able to fulfil the requirements for channel activation together with PIP_2, but in higher concentrations (10%). On the other hand, none of the lipids could activate $K_{IR}2.1$ the absence of PIP_2.[75]

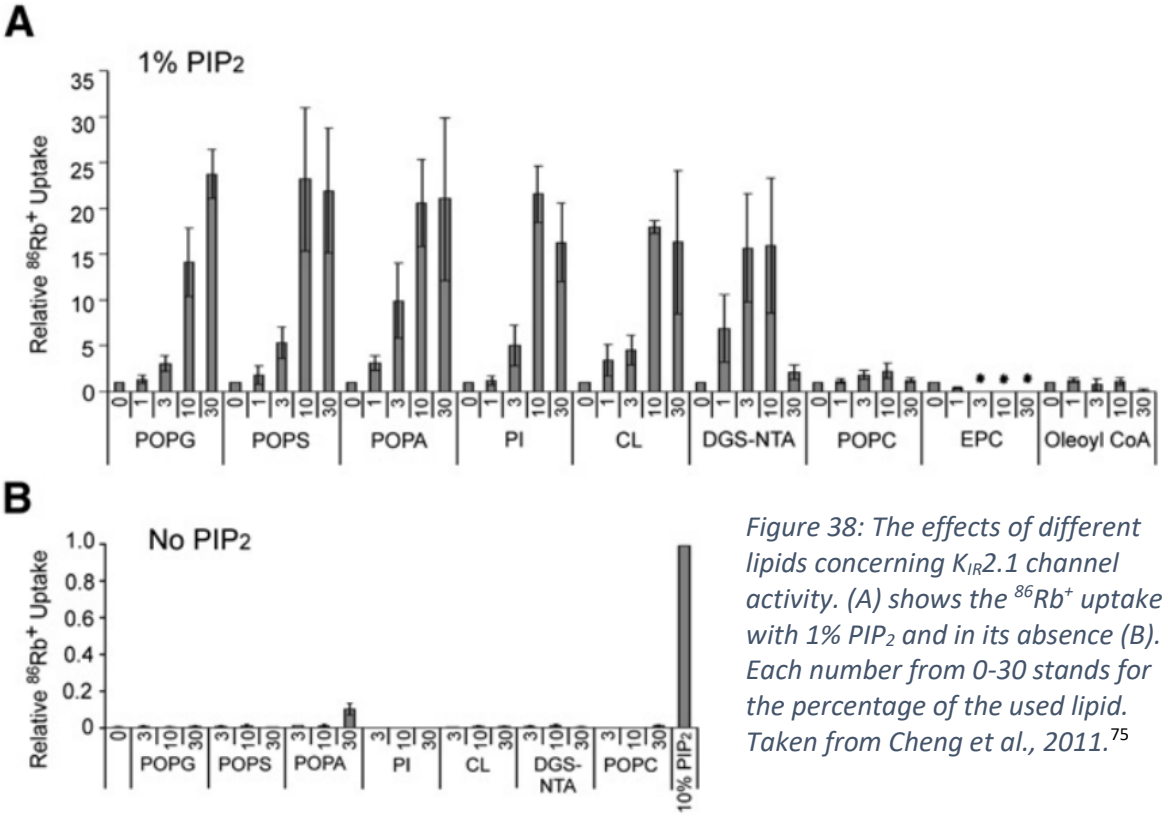

Figure 38: The effects of different lipids concerning $K_{IR}2.1$ channel activity. (A) shows the $^{86}Rb^+$ uptake with 1% PIP_2 and in its absence (B). Each number from 0-30 stands for the percentage of the used lipid. Taken from Cheng et al., 2011.[75]

Since EPC and oleoyl CoA could not activate $K_{IR}2.1$, further experiments were performed. Different concentrations of each lipid were applied to liposomes containing 1% PIP_2 and 10% POPG or only 10% PIP_2. $K_{IR}2.1$ channel activity was already inhibited at concentrations from 1-3% oleoyl CoA in liposomes with 1% PIP_2 and 10% POPG. At a 10% PIP_2 background, channels were inhibited at about 10% oleoyl CoA. EPC with its positively

charged headgroup can be seen as antagonist to secondary anionic phospholipids, since it decreases channel activity in liposomes containing 1% PIP_2 and 5% POPG.[75]

Furthermore, the effect of different PIPs on the channel activity was studied. With $PI(4,5)P_2$ as an example, it has been shown that $PI(4,5)P_2$ can act as agonist at the secondary anionic phospholipid binding site as well at higher concentrations and POPG was not needed, as presented previously. This finding led to the question, whether other PIPs fulfil the requirement of secondary lipids as well. At 1% PIP_2 concentrations, every single PIP at 10% concentration led to a $K_{IR}2.1$ channel activity, whereas PI is the most potent molecule, followed by the monophosphorylated PIPs PI(3)P, PI(4)P and PI(5)P. PIPs with two or three phosphate groups like $PI(3,4)P_2$, $PI(3,5)P_2$ and $PI(3,4,5)P_3$ showed weaker channel activation. This finding is consent with the fact that PIPs with two or more phosphate groups are more potent antagonists of PIP_2. Furthermore, this provides evidence that, although they also interfered with the primary binding site, all PIPs can act as secondary anionic phospholipid and therefore lead to a channel activation. On the other hand, if the same PIPs were applied to liposomes containing 1% $PI(4,5)P_2$ and 25% POPG, every PIP except for PI inhibited $K_{IR}2.1$, as seen in Figure 39. Concluding, the role of different PIPs other than $PI(4,5)P_2$ is dependent on the lipid composition of the bilayer. Furthermore, another experiment revealed that the extracellular side of $K_{IR}2.1$ is not involved in the activation process by PIP_2 and secondary anionic phospholipids as well. Therefore, the mutations K117A, K120A and a double mutant were introduced, and no striking changes were observed. These residues are located on the extracellular side. Contrary, mutations concerning an intracellular part were studied as well: K182Q, R189Q and K219Q. The K182Q and K189Q mutated channels showed small to no activity at all in liposomes

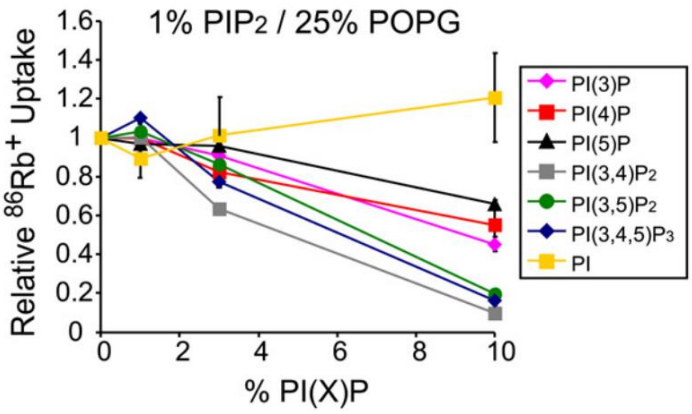

Figure 39: The inhibiting effect of different PIPs on $K_{IR}2.1$ channel activity in liposomes containing 1% $PI(4,5)P_2$ and 25% POPG. The x-axis depicts the different concentrations of each PIP. PI is the only lipid that does not lead to a channel inhibition. Taken from Cheng et al., 2011.[75]

containing 25% POPG and PIP_2 in various concentrations, whereas the K219Q mutant is activated by 10-fold higher PIP_2 concentrations in comparison to wild type channels. The results are presented in Figure 40.[75]

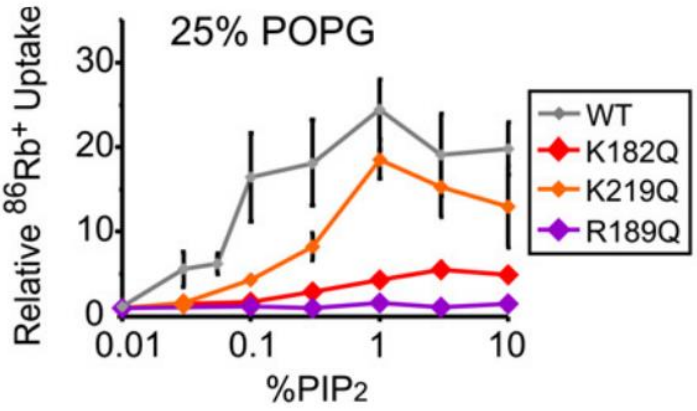

Figure 40: The effects of various mutations in $K_{IR}2.1$ channels in liposomes containing 25% POPG. R182Q and K189Q were hardly activated by PIP_2 in all concentrations. For the activation of the K219W mutant, more PIP_2 was needed. Taken from Cheng et al., 2011.[75]

7.6. Cholesterol-PIP_2 crosstalk in KIR2.x

$K_{IR}2.1$ and $K_{IR}2.3$ channels have been examined by Rosenhouse-Dantsker et al. to show how cholesterol affects the binding of PIP_2, which is presumed to occur in rather cholesterol rich parts of the plasma membrane.[76] Upon cholesterol depletion, PIP_2 seems to colocalize with other domains. One common method to quantify the strength of PIP_2 binding to K_{IR} channels is to measure the current rundown and recovery in dependence of PIP_2. For their study, neomycin was dialyzed into cells. Neomycin is known to bind to the head group of PIP_2 and keeps it from interacting with any proteins. Since PIP_2 is an important activating factor, it is reasonable that $K_{IR}2.1$ (expressed in CHO cells) reacts with a rundown upon adding neomycin. Interestingly, a depletion of cholesterol, which was managed by adding MβCD, led to a delay, which can be seen in Figure 41. A slower rundown means

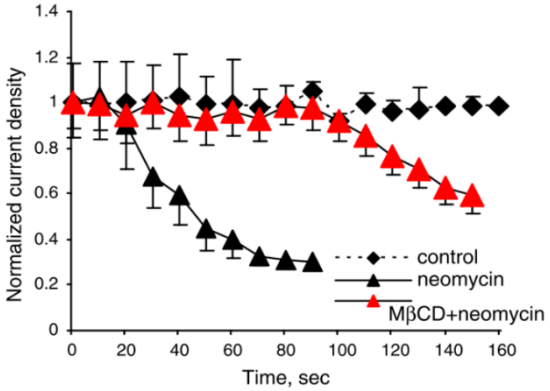

Figure 41: Rundown time of $K_{IR}2.1$. Adding neomycin leads to a rundown due to a lack of interactions with PIP_2. Cholesterol depletion causes a delay in the rundown time, which can be seen in red. Taken from Rosenhouse-Dantsker et al., 2014.[76]

stronger PIP_2 interactions. The effect is dependent on the concentration of neomycin. To show whether neomycin influenced only PIP_2 or also other molecules, another experiment was performed with specific PIP_2 antibodies instead of neomycin. Although the rundown time is generally longer with antibodies, the experiments led to the same results. An interference of MβCD to the rundown current induced by neomycin can be excluded.[76] $K_{IR}2.3$ is less sensitive to PIP_2 but leads to similar results, only with altered dose responses. Cholesterol depletion shows a stronger impact on $K_{IR}2.1$, supporting the idea of its higher cholesterol sensitivity. The most important findings are that the cholesterol sensitivity does not depend on PIP_2 and that the $K_{IR}2$-PIP_2 interaction is strengthened by cholesterol depletion. On the other hand, cholesterol enrichment does not lead to the opposite effect. One possible mechanism for the decreased channel activity in presence of cholesterol may be the binding of cholesterol to a site between two TM helices. As a result, the $K_{IR}2$-PIP_2 interaction is weakened (but never completely inhibited) and the mentioned helices cannot change to the open channel conformation easily. In absence of cholesterol, PIP_2 can bind better and the TM domain is more likely to rotate into the open channel conformation. This mechanism is presented in Figure 42.[76,77]

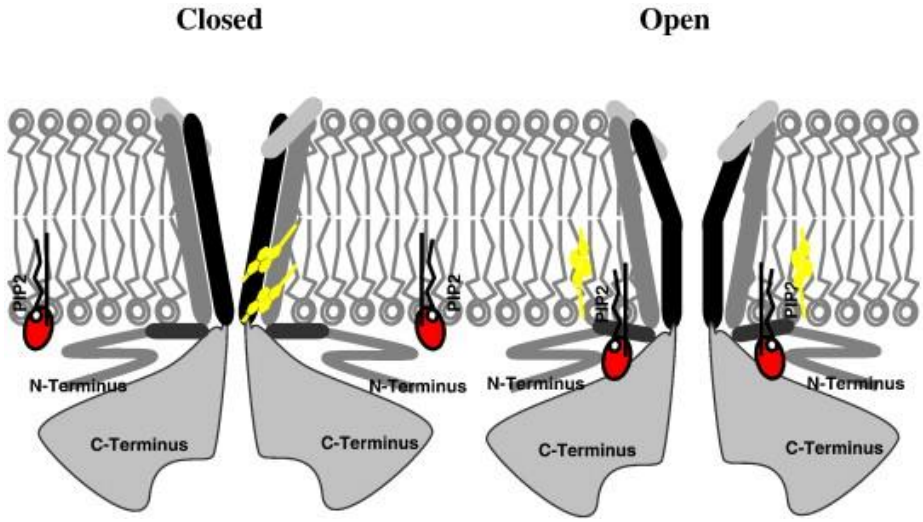

Figure 42: A model of $K_{IR}2.x$ channels in the closed (left) and open (right) state with a schematic binding of cholesterol (yellow) and PIP_2. Taken from Rosenhouse-Dantsker et al., 2014.[78]

7.7. The effects of hydrogen sulphide on K_{IR} channels depending on PIP_2

The gasotransmitter hydrogen sulphide (H_2S) is known to influence K_{IR} channels in different ways, dependent on the strength the of interaction with PIP_2. H_2S is associated with oxidative stress, necrosis, inflammation, apoptosis as well as hypoxia and acts as important cardioprotective factor during ischaemic conditions as the excitability of K_{IR} channels in cardiac cells is lowered. On the other hand, brain tissue damage due to ischaemia is worsened by H_2S because of an increased K_{IR} excitability. The endogenous synthesis of H_2S is mediated via cysteine by diverse enzymes. H_2S causes a sulfhydration to cysteine residues and thereby alters the function of proteins. Examples for these residues are Cys43 in $K_{IR}6.1$ or Cys42 in $K_{IR}6.2$. The modification of cysteine in $K_{IR}6$ channels in cardiac cells causes the blood vessels to dilate due to a hyperpolarization.
It has been observed in $K_{IR}3.2$ channels, that the cysteine residue on position 63 is located next to a positively charged lysine, which interacts with PIP_2. To study the correlation between PIP_2 binding and H_2S regulation, electrophysiological studies and MD simulations were performed by Ha et al.[78] Neither the conductance nor the localization of K_{IR} channels on the cell surface were affected by H_2S, but there occurred an increase in closed-time kinetics and a decrease in open-time kinetics, concerning $K_{IR}2$ and $K_{IR}3$ channels. Adding NaHS to a E152D mouse $K_{IR}3.2$ channel mutant led to an inhibition in the channel activity. The mutation is known to strengthen the interaction with PIP_2. Similar outcomes have been observed in S143T mutant homotetrameric $K_{IR}3.4$ channels as well as in heterotetrameric $K_{IR}3.1/K_{IR}3.4$ channels and in $K_{IR}2.3$. Both mentioned mutations were introduced to increase channel activity. Since $K_{IR}2.1$ is highly affine to PIP_2, a treatment with NaHS did not show any changes in the channel activity. On the other hand, members of the $K_{IR}6$ family reacted with an increased activity towards NaHS. Regarding $K_{IR}6.2$, an increased channel activation by NaHS has been observed when expressed with SUR2A subunits and in $K_{IR}6.2\Delta36$ channels. Latter mutation is experimentally necessary to enable $K_{IR}6.2$ channels to be expressed without SUR subunits.[78]

To assess how the NaHS treatment is affected by PIP_2, concentrations of PIP_2 were altered. A PIP_2 increase was mediated via a kinase called PIP5K, which manages the

phosphorylation of PI(4)P to PI(4,5)P_2. On the other hand, PIP_2 concentrations decreased upon adding a PIP4K blocker, namely Wortmannin. As expected, the channel activity was less reduced by NaHS when PIP_2 was present at higher concentrations and vice versa. Furthermore, the E152D mutant in $K_{IR}3.2$, which has been described previously, showed a smaller current reduction with NaHS than the wild type. This finding seems reasonable, since the mutant channels are more sensitive to PIP_2. Furthermore, introducing a second mutation (I234L) led to even higher PIP_2 sensitivities and the inhibition by NaHS was completely abolished. $K_{IR}2.3$ channels, which are known to interact intermediately with PIP_2 in comparison to other K_{IR} channels, are inhibited by NaHS at low doses. Similar to the $K_{IR}3$ channel experiments, the inhibition was lower when PIP_2 with two C-8 acyl chains was added. Results of the experiments are presented in Figure 43 and 44.[78]

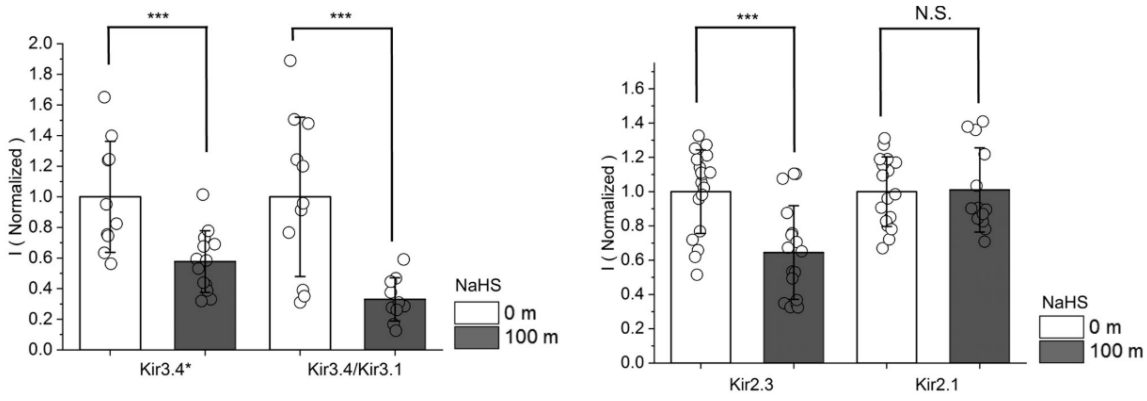

Figure 43: The effect of NaHS on S143T mutated $K_{IR}3.4$ channels, the heterotetrameric $K_{IR}3.4/K_{IR}3.1$ channel, $K_{IR}2.3$ and $K_{IR}2.1$. The normalized currents decreased in every channel in presence of NaHS except for $K_{IR}2.1$, which does not show any difference. Taken from Ha et al., 2018.[78]

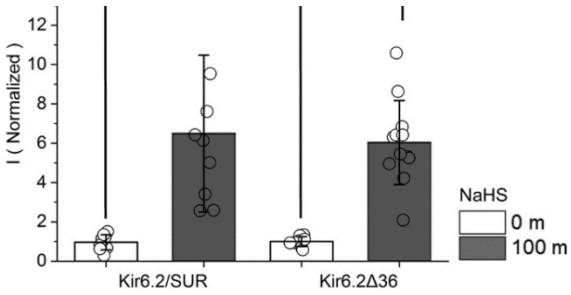

Figure 44: $K_{IR}6.2$ co-expressed with SUR2A and the Δ36 mutated $K_{IR}6.2$ channel, exposed to NaHS. Both versions of $K_{IR}6.2$ show noticeable channel activation as an increase in normalized currents could be measured. Taken from Ha et al., 2018.[78]

Furthermore, MD simulations were performed, using mouse $K_{IR}3.2$ with PIP_2 bound to it. C65, C221 and C321 were sulfhydrated in the simulation. No effects were observed, when C221 was sulfhydrated. Lysine at position 64 plays an important role in the PIP_2 binding process. First, the spatial distance between K64 and PIP_2 headgroups was

measured. Interestingly, the sulfhydration of C65 and C321 caused the distance to PIP_2 headgroups to be larger. This may provide evidence for the loss in PIP_2 binding strength, when C65 and C321 are sulfhydrated. Figure 45 shows snapshots of a simulated mouse $K_{IR}3.2$ with a PIP_2 molecule bound.[78]

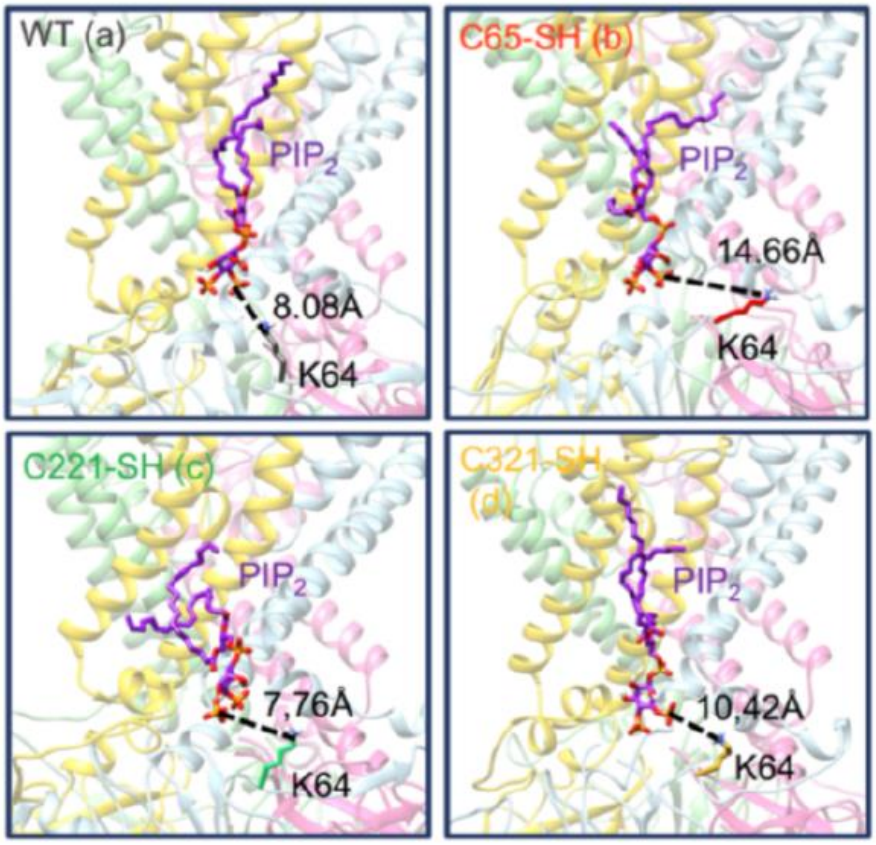

Figure 45: MD simulations of mouse $K_{IR}3.2$ with PIP_2. The spatial distance between PIP_2 headgroups and K64 was 8.08Å in wild type $K_{IR}3.2$ (black, a), 14.66Å in the C65 sulfhydrated channel (C65-SH, red, b), 7.76Å in C221-SH (green, c) and 10.42Å in C321-SH (yellow, d). Taken from Ha et al., 2018.[78]

7.8. Structural insight in PIP2 binding sites

The results of the structure alignment concerning the PIP_2 binding site are depicted in the following Figures. Only one K_{IR} subunit is shown in each visualisation for a better view. The carbons of PIP_2 are marked green, whereas the ones from the side chains, which have been described in studies earlier, are highlighted pink. Light blue residues are not experimentally proven, but they are highlighted to enable a better comparison. The rest of each K_{IR} subunit is held in grey. All presented channels are compared with the chicken $K_{IR}2.2$ channel co-crystallized with PIP_2 (3SPI).

7.8.1. Eukaryotic K_{IR} channels, 3SPI

With 3SPI, the first crystal structure of chicken $K_{IR}2.2$ co-crystallized with PIP_2 was revealed. It shows the interactions of the typical PIP_2 binding motif RWR (at positions 78-80) with PIP_2. Furthermore, three lysines (K183, K188, K189) and one arginine (186) are visualized, which are in contact with phosphate groups of PIP_2. The PKKR motif at positions 187-190 is quite common among K_{IR} channels with few exceptions ($K_{IR}3.3$, $K_{IR}5.1$, $K_{IR}6.1$, $K_{IR}6.2$ and $K_{IR}7.1$). K62 is highlighted for the comparison with the K62W mutant, which will be described later. One more amino acid, Q51, is marked as it represents the corresponding residue to K64 in $K_{IR}3.2$ (3SYA). Latter residue is known to be important for the PIP_2 binding process.[60] However, Q51 in $K_{IR}2.2$ does not seem to be critically involved since it is located further away from the PIP_2 binding site in comparison to $K_{IR}3.2$. The cytoplasmic domain is already closer to the transmembrane domain due to the PIP_2 binding. An overview of the location of PIP_2 binding sites in general is given in Figure 46. Figure 47 shows a close-up of only one subunit with the mentioned marked residues.

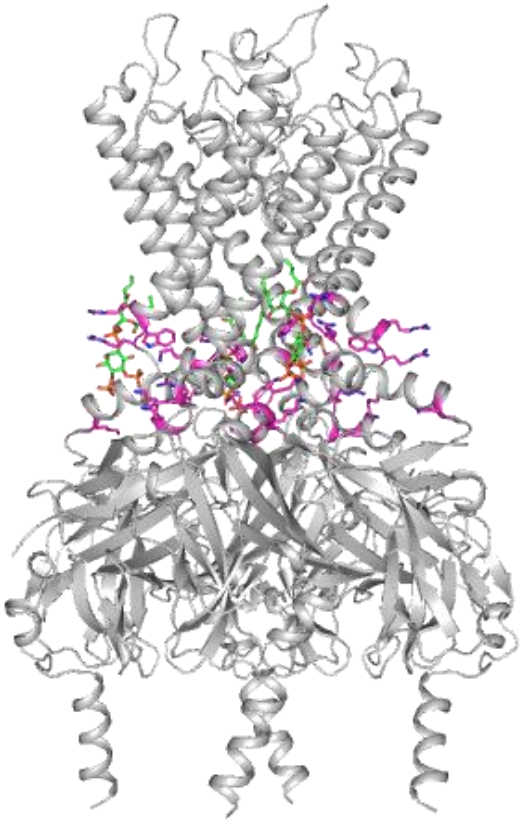

Figure 46: A biological assembly of all four subunits of chicken $K_{IR}2.2$ (3SPI) with its PIP_2 binding sites and PIP_2 co-crystallized. Visualized with PyMOL.

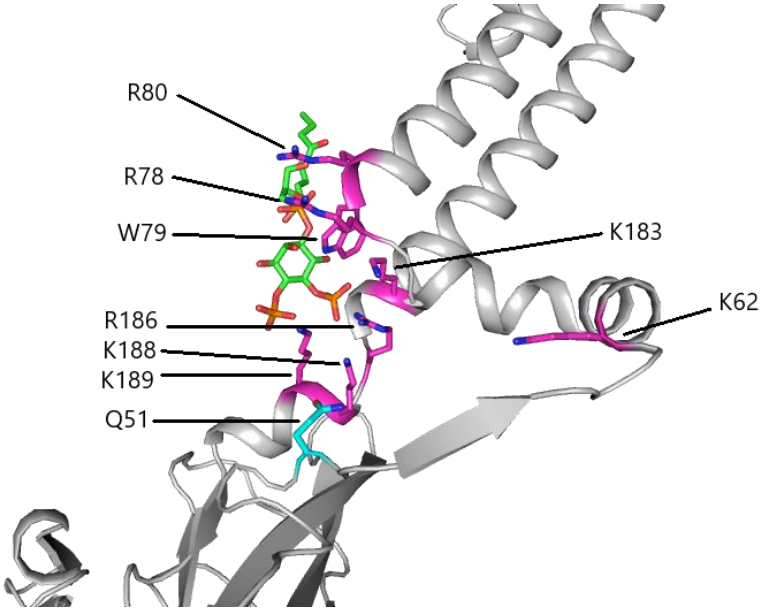

Figure 47: Chicken $K_{IR}2.2$ co-crystallized with PIP_2 (3SPI). Visualized with PyMOL.

7.8.2. 3SPJ

Concerning chicken apoK_{IR}2.2, 3SPJ reveals some differences to the PIP_2 bound structure 3SPI. An illustration can be seen in Figure 48. Again, the RWR motif (78-80), three lysines (186, 188 and 189) and one arginine (186) are highlighted and Q51 does not seem to be involved at all. Most importantly, the spatial distance between the cytoplasmic domain and the transmembrane domain is bigger without PIP_2. W79 is part of a loop and a helical structure is not properly formed yet. It is still relatively far away from TM2. Additionally, the PKKR motif also forms a loop and the residues face away from the PIP_2 binding site. A helix as it is formed in 3SPI containing the mentioned motif is not present. The binding site is not optimized for the interaction with a PIP_2 molecule yet and the necessary conditions for an opening of the channel pore are not given. The mentioned residues are still flexible and their positions are more fixed in the presence of PIP_2 or also a secondary anionic phospholipid.

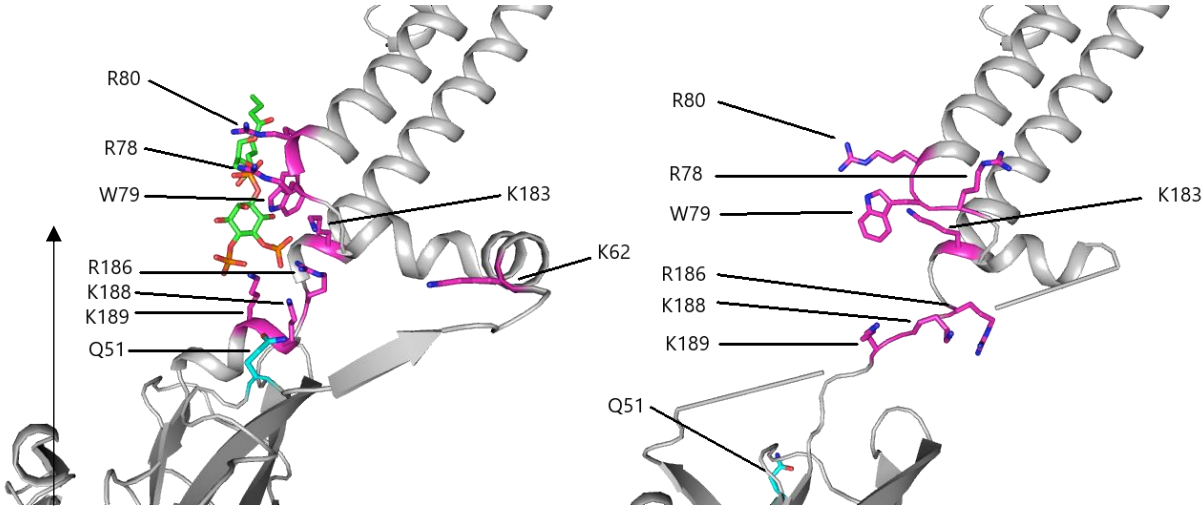

Figure 48: Chicken ApoK_{IR}2.2 (3SPJ, right) in comparison to 3SPI (left). The upwards arrow symbolizes the tethering of the cytoplasmic domain towards the transmembrane domain. Visualized with PyMOL.

7.8.3. 5KUK

The K62W mutant of ApoK_{IR}2.2 shows more similarities to the PIP_2 bound state in 3SPI than 3SPJ. Despite the absence of PIP_2, the cytoplasmic domain is relatively close to the transmembrane domain. Actually, the alignment does not show striking differences compared to 3SPI. This phenomenon is caused by the mutated lysine at position 62 in the slide helix, which is marked in Figure 49. The newly introduced tryptophan tethers the cytoplasmic domain towards the transmembrane domain as it occurs in the PIP_2 bound state. The PKKR motif and W79 are part of a helix as well. The mutation is presumed to mimic the state of K_{IR}2.2 when a secondary anionic phospholipid is bound, which prepares the channel for an optimized PIP_2 binding and leads to an open channel conformation afterwards. The PIP_2 binding sensitivity is increased (see also chapter 7.5).[53]

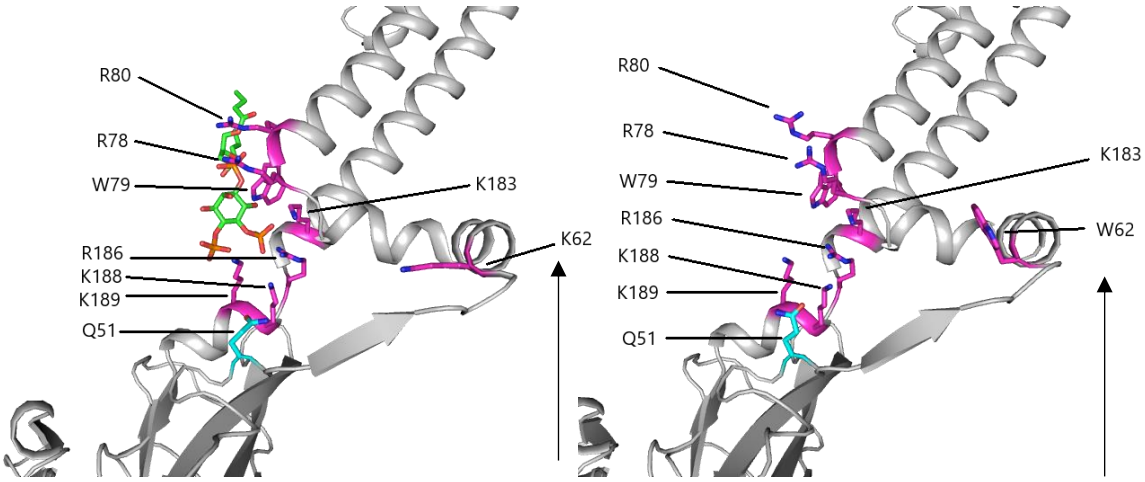

Figure 49: Chicken ApoK_{IR}2.2 with the K62W mutation (5KUK, right) leading to a membrane tethering in comparison to 3SPI (left). The upwards arrow symbolizes the tethering of the cytoplasmic domain towards the transmembrane domain. Visualized with PyMOL.

7.8.4. 3SYA

Another structure of a K_{IR} channel co-crystallized with PIP_2 is 3SYA, which is a mouse HoloK_{IR}3.2 channel (see Figure 50). The conformation is similar to 3SPI. The KWR motif (90-92) is marked again as well as three lysines surrounding a PIP_2 molecule (residues 194, 199, 200). To be mentioned, K90 in K_{IR}3.2 correlates with R78 in K_{IR}2.2. R186 in K_{IR}2.2 is similar to Q197 in K_{IR}3.2. Furthermore, the importance of the positively charged lysine at position 64 can be seen clearly in this channel as it interacts with the negative charge of a PIP_2 phosphate group. A spatial distance of 2.7Å to the nearest phosphate group of PIP_2 was measured. C65 and C321 are highlighted yellow since they have an impact on the PIP_2 binding when they are sulfhydrated (see also chapter 7.7).[78]

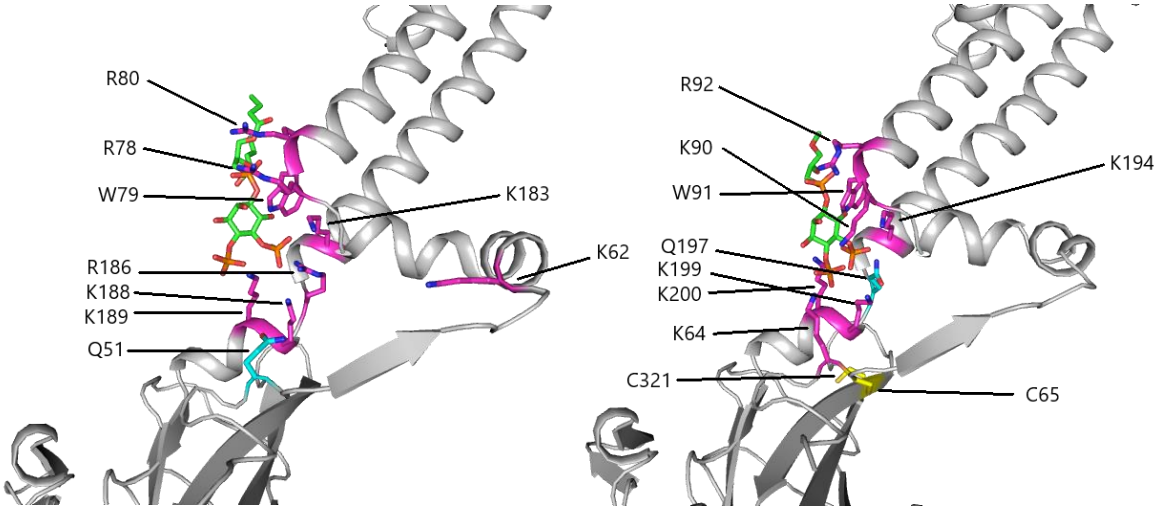

Figure 50: Mouse HoloK_{IR}3.2 (3SYA, right) in comparison to 3SPI (left). Visualized with PyMOL.

7.8.5. 6C3O

Human ApoK_{IR}6.2 (6C3O, see Figure 51) is quite different to the mentioned channels since it is surrounded by SUR subunits and its regulation is managed via numerous mechanisms. This leads to the idea that PIP_2 alone is not as important as it is in e.g. K_{IR}2 channels. The binding site differs to 3SPI as the PKKR motif is exchanged by the corresponding residues AHRR (174-177). K67 in K_{IR}6.2 refers to R78 in K_{IR}2.2 concerning the RWR motif. Furthermore, R80 (K_{IR}2.2) is replaced with proline (69, K_{IR}6.2). A helix as it occurs in 3SPI is not perfectly formed. Residues which are marked blue represent analogue amino acids to K_{IR}2.2 like K170 (K183 in K_{IR}2.2) or Q173 (R186 in K_{IR}2.2). C42 is marked yellow since its sulfhydration influences the PIP_2 binding (see also chapter 7.7).[78] Less interactions can be seen between the slide helix and the TM1. The cytoplasmic domain in general is not tethered to the transmembrane domain. The PIP_2 dependency of K_{IR}6.2 does not seem to be as important, since other mechanisms like ATP influence the channel conformation as well.

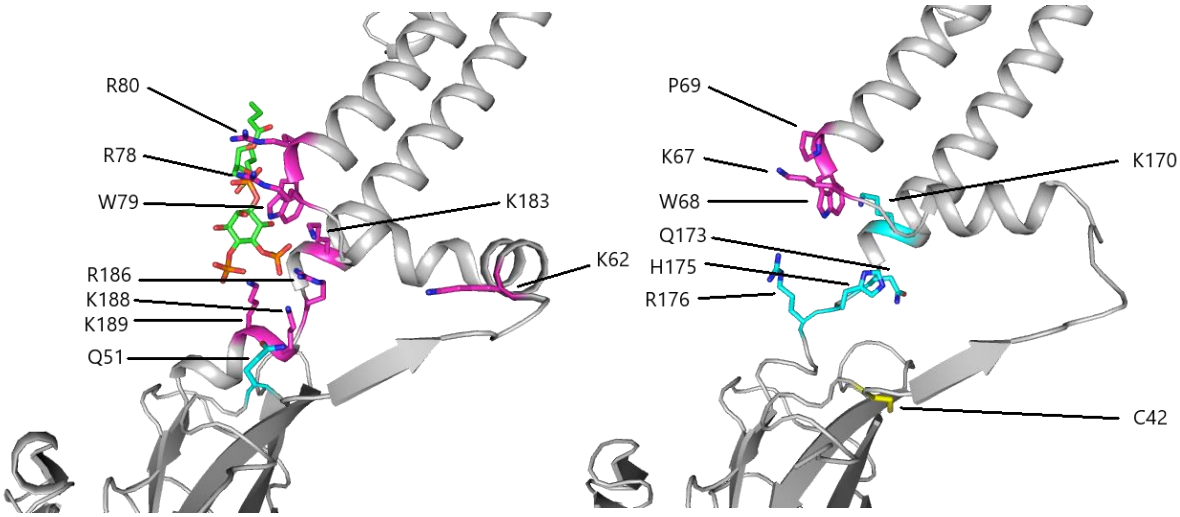

Figure 51: ApoK_{IR}6.2 (6C3O, right) in comparison to 3SPI (left). Visualized with PyMOL.

7.8.6. Bacterial K_{IR} channels, 2WLL

K_{IR}Bac1.1 as an example for bacterial K_{IR} channels is not supposed to have PIP_2 binding sites at all. Despite this fact, some basic residues nearby a binding site as it occurs in eukaryotic K_{IR} channels are marked blue in Figure 52. The location of W60 is similar to W79 in K_{IR}2.2. Furthermore, two arginine residues (148 and 151) were found. The typical PKKR motif does neither occur in K_{IR}Bac1.1 nor in K_{IR}Bac3.1. The spatial distance between the cytoplasmic and the transmembrane domain is quite small since bacterial K_{IR} channels are a few amino acids "shorter" than eukaryotic K_{IR} channels (see also chapter 7.3). What counts for K_{IR}Bac1.1 can be widely applied to K_{IR}Bac3.1 as well.

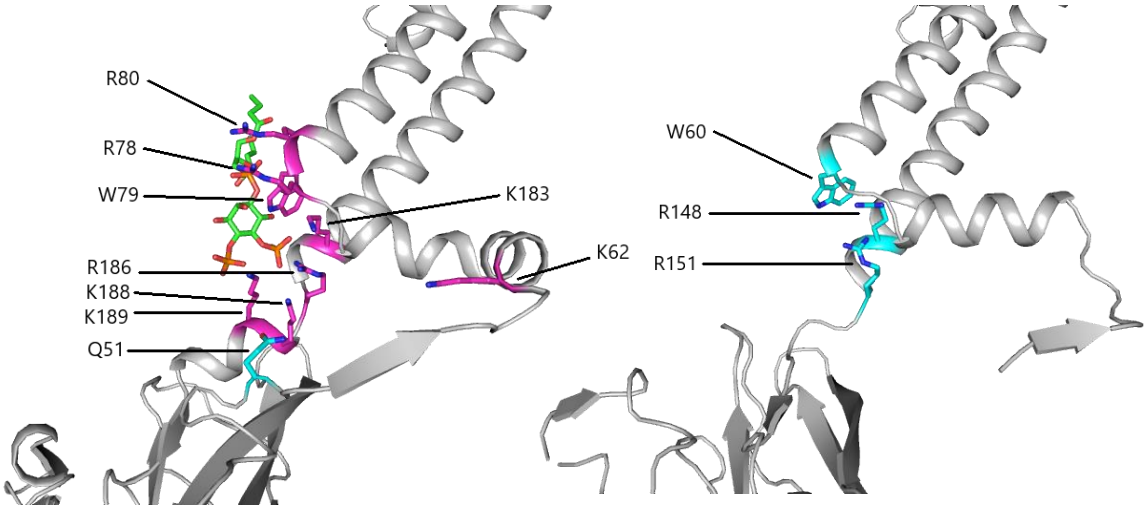

Figure 52: K_{IR}Bac1.1 (2WLL, right) in comparison to 3SPI (left). Visualized with PyMOL.

7.8.7. 4LP8 and 3ZRS

Both 4LP8 (see Figure 53) and 3ZRS (see Figure 54) represent K_{IR}Bac3.1. The crystal structures are quite identical.

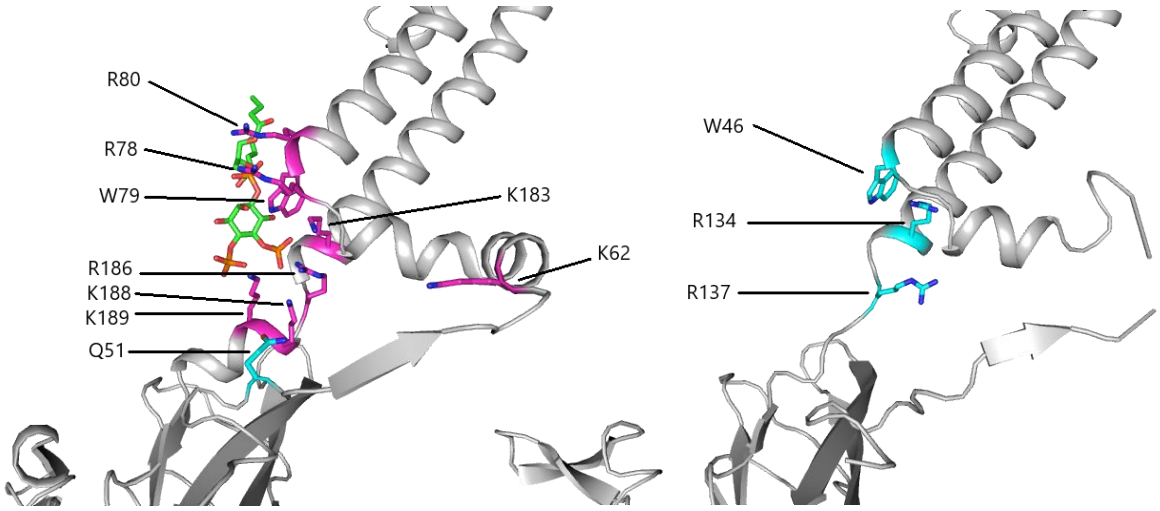

Figure 53: K_{IR}Bac3.1 (4LP8, right) in comparison to 3SPI (left). Visualized with PyMOL.

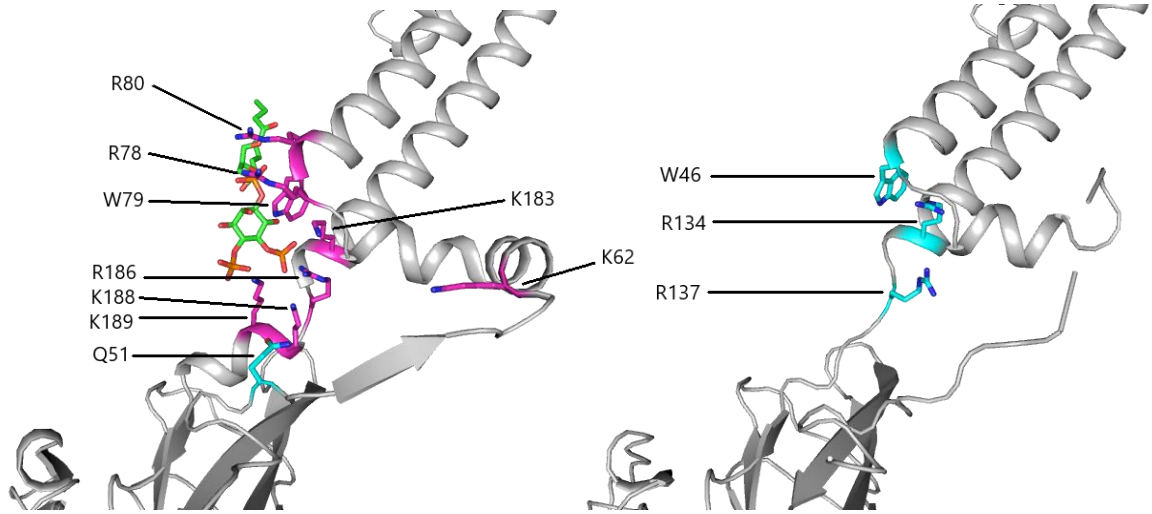

Figure 54: K_{IR}Bac3.1 (3ZRS, right) in comparison to 3SPI (left). Visualized with PyMOL.

8. Discussion

To sum up, it has been shown that inward rectifier potassium channels take over various functions in different kinds of tissues and cells and they are essential for maintaining the resting membrane potential. Mutations in K_{IR}1-7 can be silent or may also lead to severe illnesses and syndromes, when crucial amino acid residues are affected. The importance of PIP_2 has been proven in many studies (Lopes et al., 2002 or Kumar et al., 2015), as mutations altering the strength of the PIP_2 binding to K_{IR} channels leads to partially severe medical conditions like the Bartter syndrome, Andersen-Tawil syndrome or DEND.

It has been shown that various lipids have different effects on K_{IR} channels. For example, cholesterol inhibits most of the channels. This phenomenon has been thoroughly observed in *in vivo* and *in vitro* studies (Fang et al., 2006) as well as in simulations (Barbera et al., 2018). However, GIRK channels may also be up-regulated by cholesterol. Not every channel shows the same sensitivity, which can be at least partially explained by the co-localization with caveolin and the composition of lipid rafts (Levitan, 2009). A structural alignment concerning putative cholesterol binding sites in K_{IR} channels shows that mainly lipophilic residues are involved. Furthermore, the location of the binding sites is quite comparable among single families including bacterial K_{IR} channels (Fürst et al., 2014). However, experimental data is still lacking to provide further insight.

PIP_2 acts somewhat opposite to cholesterol to several mammalian K_{IR} channels; however, not all K_{IR} channels show the same PIP_2 sensitivity. The most important PIP_2 sensitive K_{IR} channel family is K_{IR}2. Together with a secondary anionic phospholipid, the PIP_2 binding to its primary site leads to a conformational change of the cytoplasmic domain towards the transmembrane domain and to an opening of the channel pore (Lee et al., 2013). It has been shown that the channel conformation of the K_{IR}2.2 K62W mutant is quite similar to the PIP_2 bound state, even when PIP_2 is not present (Lee et al., 2016). The mutant seems to mimic the role of a secondary anionic phospholipid, by anchoring the cytoplasmic domain to the membrane. Furthermore, some differences in the crucial residues concerning the binding site were revealed. Differences between single K_{IR} channels were shown in a structural alignment of different K_{IR} channels, crystallized with and without PIP_2 bound. For example, the locations and directions of various residues

were altered in the presence and the absence of PIP_2. $K_{IR}6.2$, which is known from experiments to be less sensitive to PIP_2 than $K_{IR}2$ channels, does not seem to be influenced by PIP_2 as much as e.g. $K_{IR}2$ channels since other mechanisms like ATP, Mg^{2+} or the influence of the adjacent transporters with unrevealed mechanisms of action play important roles as well.

Furthermore, some differences in the crucial residues concerning the PIP_2 binding site were revealed. An example is K64 in $K_{IR}3.2$, which has been described to be crucially involved in the PIP_2 binding process (Qiao et al. in 2020). The measured spatial distance to the nearest phosphate group of PIP_2 is 2.7Å. The corresponding residue Q51 in $K_{IR}2.2$ is about 9.3Å away from the nearest phosphate group of a matching co-crystallized PIP_2 molecule and does not seem to be as important as K64 in $K_{IR}3.2$.

It has been shown, that K_{IR} channels do not react equally to different types of phosphatidylinositolphosphates. $PI(4,5)P_2$ seems to be the most important PIP to K_{IR} channels. One phosphate group like in PI(4)P is insufficient to activate K_{IR} channels to their maximum (D'Avanzo et al., 2010). Other channels like members of the $K_{IR}3$ family react with a channel pore opening towards the binding of a $PI(3,4,5)P_3$ molecule similar to $PI(4,5)P_2$ (Liu et al., 2019).

Bacterial K_{IR} channels are inhibited by PIP_2 (D'Avanzo et al., 2010). This phenomenon seems reasonable, when the sequences and crystal structures of mammalian and bacterial K_{IR} channels are aligned. The difference can be seen as an evolvement of eukaryotic cells over the time and an adaption to the altered composition of the lipid bilayer.

Despite numerous publications on the field of lipid interactions with K_{IR} channels, there are still many unanswered questions and large research possibilities. Although plenty of distinct mutations have already been identified and many diseases and syndromes are known, in many cases approaches of medical treatments are still missing, especially for orphan medical conditions. Further investigations are necessary and the common modern research methods enable a wide range of possibilities.

9. Appendix

9.1. References

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Ich habe mich bemüht, sämtliche Inhaber*innen der Bildrechte ausfindig zu machen und ihre Zustimmung zur Verwendung der Bilder in dieser Arbeit eingeholt. Sollte dennoch eine Urheberrechtsverletzung bekannt werden, ersuche ich um Meldung bei mir.

9.2. Sequence alignment

The first yellow part depicts TM1, the second TM2. The H5 region is marked light grey. Methionine on the beginning of each sequence is held in olive. The sequence for the selectivity filter is depicted in light green while S147 of $K_{IR}2.4$ is marked violet for clarity reasons. The site that is important for the gating mechanism via Mg^{2+} and polyamines is marked dark grey. M125 in $K_{IR}7.1$, which results in important properties, is depicted in light red to show the difference to all the other K_{IR} channels having arginine at the equivalent positions. The three amino acid sequence in dark blue shows the interaction site of 1' phosphate of PIP_2. The two motifs in dark red show residues, that do not appear in bacterial K_{IR} channels. Residues highlighted in light blue are described throughout this work and are associated with PIP_2, pink with cholesterol. Sequences in dark red show the residues that do not occur in bacterial K_{IR} channels (see chapter 7.3)

```
KIR1.1 /001-046    --------------MNASSRNVFDTLIRVLTESMFKHLRKWVVTRFFGHSRQRARLVSKD
KIR2.1 /001-051    ---------MGSVRTNRYSIVSSEEDGMKLATMAVANGFGNGKSKVHTRQQCRSRFVKKD
KIR2.2 /001-050    ----------MTAASRANPYSIVSSEEDGLHLVTMSGANGFGNGKVHTRRRCRNRFVKKN
KIR2.3 /001-025    ------------------------------------MHGHSRNGQAHVPRRKRRNRFVKKN
KIR2.4 /001-056    ----MGLARALRRLSGALDSGDSRAGDEEEAGPGLCRNGWAPAPVQSPVGRRRGRFVKKD
KIR2.6 /001-050    ----------MTAASRANPYSIVSLEEDGLHLVTMSGANGFGNGKVHTRRRCRNRFVKKN
KIR3.1 /001-050    ----------MSALRRKFGDDYQVVTTSSSGSGLQPQGPGQDPQQQLVPKKKRQRFVDKN
KIR3.2 /001-060    MAKLTESMTNVLEGDSMDQDVESPVAIHQPKLPKQARDDLPRHISRDRTKRKIQRYVRKD
KIR3.3 /001-028    --------------------------------MAQENAAFSPGQEEPPRRRGRQRYVEKD
KIR3.4 /001-057    ---MAGDSRNAMNQDMEIGVTPWDPKKIPKQARDYVPIATDRTRLLAEGKKPRQRYMEKS
KIR4.1 /001-034    ---------------------------MTSVAKVYYSQTTQTESRPLMGPGIRRRRVLTKD
KIR4.2 /001-033    ---------------------------MDAIHIGMSSTPLVKHTAGAGLKANRPRVMSKS
KIR5.1 /001-040    --------------------MSYYGSSYHIINADAKYPGYPPEHIIAEKRRARRRLLHKD
KIR6.1 /001-040    --------------------MLARKSIIPEEYVLARIAAENLRKPRIRDRLPKARFIAKS
KIR6.2 /001-039    ---------------------MLSRKGIIPEEYVLTRLAEDPAEPRYRARQRRARFVSKK
KIR7.1 /001-024    ------------------------------------MDSSNCKVIAPLLSQRYRRMVTKD

KIR1.1 /047-106    GRCNIEFGNVEAQSRFIFFVDIWTTVLDLKWRYKMTIFITAFLGSWFFFGLLWYAVAYIH
KIR2.1 /052-110    GHCNVQFINVGEKG-QRYLADIFTTCVDIRWRWMLVIFCLAFVLSWLFFGCVFWLIALLH
KIR2.2 /051-109    GQCNIEFANMDEKS-QRYLADMFTTCVDIRWRYMLLIFSLAFLASWLLFGIIFWVIAVAH
KIR2.3 /026-084    GQCNVYFANLSNKS-QRYMADIFTTCVDTRWRYMLMIFSAAFLVSWLFFGLLFWCIAFFH
KIR2.4 /057-115    GHCNVRFVNLGGQG-ARYLSDLFTTCVDVRWRWMCLLFSCSFLASWLLFGLAFWLIASLH
KIR2.6 /051-109    GQCNIAFANMDEKS-QRYLADMFTTCVDIRWRYMLLIFSLAFLASWLLFGVIFWVIAVAH
KIR3.1 /051-109    GRCNVQHGNLGSET-SRYLSDLFTTLVDLKWRWNLFIFILTYTVAWLFMASMWWVIAYTR
KIR3.2 /061-118    GKCNVHHGNV-RET-YRYLTDIFTTLVDLKWRFNLLIFVMVYTVTWLFFGMIWWLIAYIR
KIR3.3 /029-086    GRCNVQQGNV-RET-YRYLTDLFTTLVDLQWRLSLLFFVLAYALTWLFFGAIWWLIAYGR
KIR3.4 /058-115    GKCNVHHGNV-QET-YRYLSDLFTTLVDLKWRFNLLVFTMVYTVTWLFFGFIWWLIAYIR
KIR4.1 /035-093    GRSNVRMEHIADKR-FLYLKDLWTTFIDMQWRYKLLLFSATFAGTWFLFGVVWYLVAVAH
KIR4.2 /034-092    GHSNVRIDKVDGIY-LLYLQDLWTTVIDMKWRYKLTLFAATFVMTWFLFGVIYYAIAFIH
KIR5.1 /041-099    GSCNVYFKHIFGEW-GSYVVDIFTTLVDTKWRHMFVIFSLSYILSWLIFGSVFWLIAFHH
KIR6.1 /041-098    GACNLAHKNI-REQ-GRFLQDIFTTLVDLKWRHTLVIFTMSFLCSWLLFAIMWWLVAFAH
KIR6.2 /040-097    GNCNVAHKNI-REQ-GRFLQDVFTTLVDLKWRHTLLIFTMSFLCSWLLFAMAWWLIAFAH
KIR7.1 /025-082    GHSTLQMDGA-QRG-LAYLRDAWGILMDMRWRWMMLVFSASFVVHWLVFAVLWYVLAEMN
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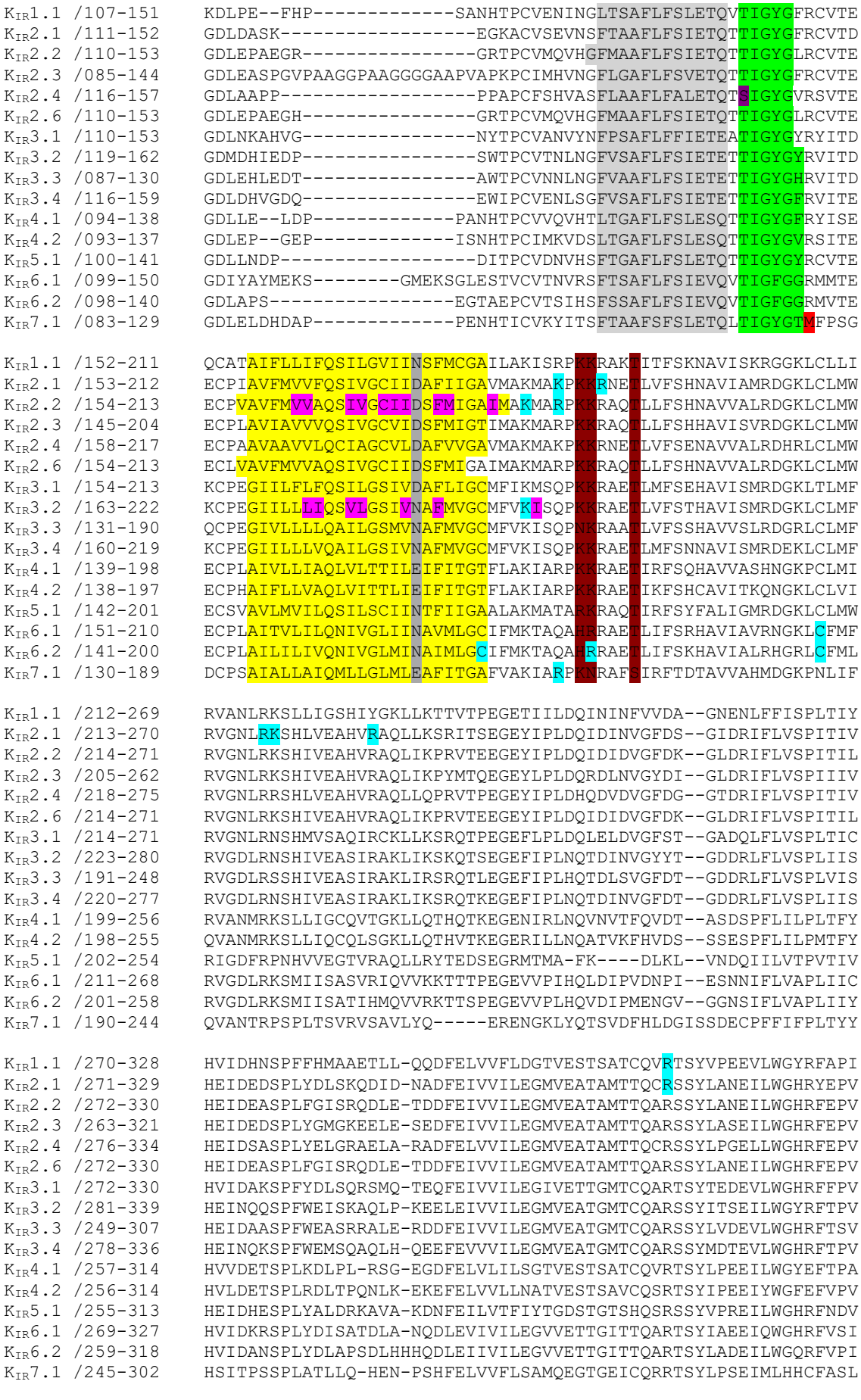
KIR1.1 /107-151 KDLPE--FHP----------SANHTPCVENINGLTSAFLFSLETQVTIGYGFRCVTE
KIR2.1 /111-152 GDLDASK----------EGKACVSEVNSFTAAFLFSIETQTTIGYGFRCVTD
KIR2.2 /110-153 GDLEPAEGR----------GRTPCVMQVHGFMAAFLFSIETQTTIGYGLRCVTE
KIR2.3 /085-144 GDLEASPGVPAAGGPAAGGGGAAPVAPKPCIMHVNGFLGAFLFSVETQTTIGYGFRCVTE
KIR2.4 /116-157 GDLAAPP----------PPAPCFSHVASFLAAFLFALETQTSIGYGVRSVTE
KIR2.6 /110-153 GDLEPAEGH----------GRTPCVMQVHGFMAAFLFSIETQTTIGYGLRCVTE
KIR3.1 /110-153 GDLNKAHVG----------NYTPCVANVYNFPSAFLFFIETEATIGYGYRYITD
KIR3.2 /119-162 GDMDHIEDP----------SWTPCVTNLNGFVSAFLFSIETETTIGYGYRVITD
KIR3.3 /087-130 GDLEHLEDT----------AWTPCVNNLNGFVAAFLFSIETETTIGYGHRVITD
KIR3.4 /116-159 GDLDHVGDQ----------EWIPCVENLSGFVSAFLFSIETETTIGYGFRVITE
KIR4.1 /094-138 GDLLE--LDP----------PANHTPCVVQVHTLTGAFLFSLESQTTIGYGFRYISE
KIR4.2 /093-137 GDLEP--GEP----------ISNHTPCIMKVDSLTGAFLFSLESQTTIGYGVRSITE
KIR5.1 /100-141 GDLLNDP----------DITPCVDNVHSFTGAFLFSLETQTTIGYGYRCVTE
KIR6.1 /099-150 GDIYAYMEKS--------GMEKSGLESTVCVTNVRSFTSAFLFSIEVQVTIGFGGRMMTE
KIR6.2 /098-140 GDLAPS----------EGTAEPCVTSIHSFSSAFLFSIEVQVTIGFGGRMVTE
KIR7.1 /083-129 GDLELDHDAP----------PENHTICVKYITSFTAAFSFSLETQLTIGYGTMFPSG
KIR1.1 /152-211 QCATAIFLLIFQSILGVIINSFMCGAILAKISRPKKRAKTITFSKNAVISKRGGKLCLLI
KIR2.1 /153-212 ECPIAVFMVVFQSIVGCIIDAFIIGAVMAKMAKPKKRNETLVFSHNAVIAMRDGKLCLMW
KIR2.2 /154-213 ECPVAVFMVVAQSIVGCIIDSFMIGAIMAKMARPKKRAQTLLFSHNAVVALRDGKLCLMW
KIR2.3 /145-204 ECPLAVIAVVVQSIVGCVIDSFMIGTIMAKMARPKKRAQTLLFSHHAVISVRDGKLCLMW
KIR2.4 /158-217 ECPAAVAAVVLQCIAGCVLDAFVVGAVMAKMAKPKKRNETLVFSENAVVALRDHRLCLMW
KIR2.6 /154-213 ECLVAVFMVVAQSIVGCIIDSFMIGAIMAKMARPKKRAQTLLFSHNAVVALRDGKLCLMW
KIR3.1 /154-213 KCPEGIILFLFQSILGSIVDAFLIGCMFIKMSQPKKRAETLMFSEHAVISMRDGKLTLMF
KIR3.2 /163-222 KCPEGIILLLIQSVLGSIVNAFMVGCMFVKISQPKKRAETLVFSTHAVISMRDGKLCLMF
KIR3.3 /131-190 QCPEGIVLLLLQAILGSMVNAFMVGCMFVKISQPNKRAATLVFSSHAVVSLRDGRLCLMF
KIR3.4 /160-219 KCPEGIILLLVQAILGSIVNAFMVGCMFVKISQPKKRAETLMFSNNAVISMRDEKLCLMF
KIR4.1 /139-198 ECPLAIVLLIAQLVLTTILEIFITGTFLAKIARPKKRAETIRFSQHAVVASHNGKPCLMI
KIR4.2 /138-197 ECPHAIFLLVAQLVITTLIEIFITGTFLAKIARPKKRAETIKFSHCAVITKQNGKLCLVI
KIR5.1 /142-201 ECSVAVLMVILQSILSCIINTFIIGAALAKMATARKRAQTIRFSYFALIGMRDGKLCLMW
KIR6.1 /151-210 ECPLAITVLILQNIVGLIINAVMLGCIFMKTAQAHRRAETLIFSRHAVIAVRNGKLCFMF
KIR6.2 /141-200 ECPLAILILIVQNIVGLMINAIMLGCIFMKTAQAHRRAETLIFSKHAVIALRHGRLCFML
KIR7.1 /130-189 DCPSAIALLAIQMLLGLMLEAFITGAFVAKIARPKNRAFSIRFTDTAVVAHMDGKPNLIF
KIR1.1 /212-269 RVANLRKSLLIGSHIYGKLLKTTVTPEGETIILDQININFVVDA--GNENLFFISPLTIY
KIR2.1 /213-270 RVGNLRKSHLVEAHVRAQLLKSRITSEGEYIPLDQIDINVGFDS--GIDRIFLVSPITIV
KIR2.2 /214-271 RVGNLRKSHIVEAHVRAQLIKPRVTEEGEYIPLDQIDIDVGFDK--GLDRIFLVSPITIL
KIR2.3 /205-262 RVGNLRKSHIVEAHVRAQLIKPYMTQEGEYLPLDQRDLNVGYDI--GLDRIFLVSPIIIV
KIR2.4 /218-275 RVGNLRRSHLVEAHVRAQLLQPRVTPEGEYIPLDHQDVDVGFDG--GTDRIFLVSPITIV
KIR2.6 /214-271 RVGNLRKSHIVEAHVRAQLIKPRVTEEGEYIPLDQIDIDVGFDK--GLDRIFLVSPITIL
KIR3.1 /214-271 RVGNLRNSHMVSAQIRCKLLKSRQTPEGEFLPLDQLELDVGFST--GADQLFLVSPLTIC
KIR3.2 /223-280 RVGDLRNSHIVEASIRAKLIKSKQTSEGEFIPLNQTDINVGYYT--GDDRLFLVSPLIIS
KIR3.3 /191-248 RVGDLRSSHIVEASIRAKLIRSRQTLEGEFIPLHQTDLSVGFDT--GDDRLFLVSPLVIS
KIR3.4 /220-277 RVGDLRNSHIVEASIRAKLIKSRQTKEGEFIPLNQTDINVGFDT--GDDRLFLVSPLIIS
KIR4.1 /199-256 RVANMRKSLLIGCQVTGKLLQTHQTKEGENIRLNQVNVTFQVDT--ASDSPFLILPLTFY
KIR4.2 /198-255 QVANMRKSLLIQCQLSGKLLQTHVTKEGERILLNQATVKFHVDS--SSESPFLILPMTFY
KIR5.1 /202-254 RIGDFRPNHVVEGTVRAQLLRYTEDSEGRMTMA-FK----DLKL--VNDQIILVTPVTIV
KIR6.1 /211-268 RVGDLRKSMIISASVRIQVVKKTTTPEGEVVPIHQLDIPVDNPI--ESNNIFLVAPLIIC
KIR6.2 /201-258 RVGDLRKSMIISATIHMQVVRKTTSPEGEVVPLHQVDIPMENGV--GGNSIFLVAPLIIY
KIR7.1 /190-244 QVANTRPSPLTSVRVSAVLYQ-----ERENGKLYQTSVDFHLDGISSDECPFFIFPLTYY
KIR1.1 /270-328 HVIDHNSPFFHMAAETLL-QQDFELVVFLDGTVESTSATCQVRTSYVPEEVLWGYRFAPI
KIR2.1 /271-329 HEIDEDSPLYDLSKQDID-NADFEIVVILEGMVEATAMTTQCRSSYLANEILWGHRYEPV
KIR2.2 /272-330 HEIDEASPLFGISRQDLE-TDDFEIVVILEGMVEATAMTTQARSSYLANEILWGHRFEPV
KIR2.3 /263-321 HEIDEDSPLYGMGKEELE-SEDFEIVVILEGMVEATAMTTQARSSYLASEILWGHRFEPV
KIR2.4 /276-334 HEIDSASPLYELGRAELA-RADFELVVILEGMVEATAMTTQCRSSYLPGELLWGHRFEPV
KIR2.6 /272-330 HEIDEASPLFGISRQDLE-TDDFEIVVILEGMVEATAMTTQARSSYLANEILWGHRFEPV
KIR3.1 /272-330 HVIDAKSPFYDLSQRSMQ-TEQFEIVVILEGIVETTGMTCQARTSYTEDEVLWGHRFFPV
KIR3.2 /281-339 HEINQQSPFWEISKAQLP-KEELEIVVILEGMVEATGMTCQARSSYITSEILWGYRFTPV
KIR3.3 /249-307 HEIDAASPFWEASRRALE-RDDFEIVVILEGMVEATGMTCQARSSYLVDEVLWGHRFTSV
KIR3.4 /278-336 HEINQKSPFWEMSQAQLH-QEEFEVVVILEGMVEATGMTCQARSSYMDTEVLWGHRFTPV
KIR4.1 /257-314 HVVDETSPLKDLPL-RSG-EGDFELVLILSGTVESTSATCQVRTSYLPEEILWGYEFTPA
KIR4.2 /256-314 HVLDETSPLRDLTPQNLK-EKEFELVVLLNATVESTSAVCQSRTSYIPEEIYWGFEFVPV
KIR5.1 /255-313 HEIDHESPLYALDRKAVA-KDNFEILVTFIYTGDSTGTSHQSRSSYVPREILWGHRFNDV
KIR6.1 /269-327 HVIDKRSPLYDISATDLA-NQDLEVIVILEGVVETTGITTQARTSYIAEEIQWGHRFVSI
KIR6.2 /259-318 HVIDANSPLYDLAPSDLHHHQDLEIIVILEGVVETTGITTQARTSYLADEILWGQRFVPI
KIR7.1 /245-302 HSITPSSPLATLLQ-HEN-PSHFELVVFLSAMQEGTGEICQRRTSYLPSEIMLHHCFASL

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K_IR1.1 /329-386    VSKTKEGKYRVDFHNFSKTVEVE-TPHCAM-CLYNEKDVRARMKRGYDNPNFILSEVNET
K_IR2.1 /330-388    LFE-EKHYYKVDYSRFHKTYEVPNTPLCSARDLAEKKYILSNANSFCYENEVALTSKEED
K_IR2.2 /331-389    LFE-EKNQYKIDYSHFHKTYEVPSTPRCSAKDLVENKFLLPSANSFCYENELAFLSRDEE
K_IR2.3 /322-380    VFE-EKSHYKVDYSRFHKTYEVAGTPCCSARELQESKITVLPAPPPPPSAFCYENELALM
K_IR2.4 /335-393    LFQ-RGSQYEVDYRHFHRTYEVPGTPVCSAKELDERAEQASHSLKSSFPGSLTAFCYENE
K_IR2.6 /331-389    LFE-EKNQYKIDYSHFHKTYEVPSTPRCSAKDLVENKFLLPSANSFCYENELAFLSRDEE
K_IR3.1 /331-387    ISL-EEGFFKVDYSQFHATFEVP-TPPYSVKEQEEMLLMSSPLIAPAITNSKE-RHNSVE
K_IR3.2 /340-397    LTL-EDGFYEVDYNSFHETYETS-TPSLSAKELAELASRAELPLSWSVSSKLNQHAELET
K_IR3.3 /308-365    LTL-EDGFYEVDYASFHETFEVP-TPSCSARELAEAAARLDAHLYWSIPSRLDEKVEEEG
K_IR3.4 /337-394    LTL-EKGFYEVDYNTFHDTYETN-TPSCCAKELAEMKREGRLLQYLPSPPLLGGCAEAGL
K_IR4.1 /315-373    ISLSASGKYIADFSLFDQVVKVA-SPSGLRDSTVRYGDPEKLKLEESLREQAEKEGSALS
K_IR4.2 /315-373    VSLSKNGKYVADFSQFEQIRKSP-DCTFYCADSEKQQLEEKYRQEDQRERELRTLLLQQS
K_IR5.1 /314-371    LEV-KRKYYKVNCLQFEGSVEVY-APFCSAKQLDWKDQQLHIEKAPPVRESCTSDTKARR
K_IR6.1 /328-384    VTE-EEGVYSVDYSKFGNTVKVA-APRCSARELDEKPSILIQTLQKSELSHQNSLR-KRN
K_IR6.2 /319-376    VAE-EDGRYSVDYSKFGNTIKVP-TPLCTARQLDEDHSLLEALTLASARGPLRKRSVPMA
K_IR7.1 /303-360    LTRGSKGEYQIKMENFDKTVPEF-PTPLVSKSPNRTDLDIHINGQSIDNFQISETGLTE-

K_IR1.1 /387-391    DDTKM----------------------------------------------------
K_IR2.1 /389-427    DSENGVPESTSTDTPPDIDLHNQASVPLEPRPLRRESEI--------------------
K_IR2.2 /390-433    DEADGDQDGRSRDGLSPQARHDFDRLQAGGGVLEQRPYRRESEI----------------
K_IR2.3 /381-439    SQEEEEMEEEAAAAAAVAAGLGLEAGSKEEAGIIRMLEFGSHLDLE-RMQASLPLDNISY
K_IR2.4 /394-436    LALSCCQEEDEDDETEEGNGVETEDGAASPRVLTPTLALTLPP-----------------
K_IR2.6 /390-433    DEADGDQDGRSRDGLSPQARHDFDRLQAGGGVLEQRPYRRGSEI----------------
K_IR3.1 /388-447    CLDGLDDITTKLPSKLQKITGREDFPKKLLRMSSTTSEKAYSLGDLPMKLQRISSVPGNS
K_IR3.2 /398-423    EEEEKNLEEQTERNGDVANLENESKV---------------------------------
K_IR3.3 /366-393    AGEGAGGEAGADKEQNGCLPPPESESKV-------------------------------
K_IR3.4 /395-419    DAEAEQNEEDEPKGLGGSREARGSV-----------------------------------
K_IR4.1 /374-379    VRISNV-------------------------------------------------------
K_IR4.2 /374-375    NV---------------------------------------------------------
K_IR5.1 /372-418    RSFSAVAIVSSCENPEETTTSATHEYRETPYQKALLTLNRISVESQM-------------
K_IR6.1 /385-424    SM-RRNNSMRRNNSIRRNNSSLMVPKVQFMTPEGNQNTSES--------------------
K_IR6.2 /377-390    KAKPKFSISPDSLS-----------------------------------------------
K_IR7.1 /-------    -------------------------------------------------------------

K_IR1.1 /-------    ----------------------------------------------------------
K_IR2.1 /-------    ----------------------------------------------------------
K_IR2.2 /-------    ----------------------------------------------------------
K_IR2.3 /440-445    RRESAI-----------------------------------------------------
K_IR2.4 /-------    ----------------------------------------------------------
K_IR2.6 /-------    ----------------------------------------------------------
K_IR3.1 /448-501    EEKLVSKTTKMLSDPMSQSVADLPPKLQKMAGGAARMEGNLPAKLRKMNSDRFT
K_IR3.2 /-------    ----------------------------------------------------------
K_IR3.3 /-------    ----------------------------------------------------------
K_IR3.4 /-------    ----------------------------------------------------------
K_IR4.1 /-------    ----------------------------------------------------------
K_IR4.2 /-------    ----------------------------------------------------------
K_IR5.1 /-------    ----------------------------------------------------------
K_IR6.1 /-------    ----------------------------------------------------------
K_IR6.2 /-------    ----------------------------------------------------------
K_IR7.1 /-------    ----------------------------------------------------------
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