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A comprehensive study of the voltage gated potassium channel Kv4.3

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A comprehensive study of the voltage-gated potassium channel Kv4.3

From functional analysis to molecular dynamics
modelling

Claudio Tiecher

A comprehensive study of the voltage-gated potassium channel Kv4.3 -
From functional analysis to molecular dynamics modelling

The research presented in this Ph.D. dissertation was conducted at the Section of Molecular Neurobiology, Department of Biomedical Sciences of Cells and Systems at the University Medical Center Groningen and the laboratory of Integrative Physiology at Osaka University.

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From functional analysis to molecular dynamics
modelling

PhD thesis

to obtain the degree of PhD at the
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the decision by the College of Deans.

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Chapter 1

Introduction

1 The action potential

Neuronal cells encode information in the form of action potentials with different shapes, patterns, and frequencies. An action potential occurs when the membrane potential reaches the threshold potential at the initial segment of the axon, namely the axon hillock. From the axon hillock, the action potential efficiently propagates forward until it reaches the axon terminal. Here, the electrical signal transfers to the next neuron via the release of neurotransmitters. Whether the membrane potential reaches the threshold potential depends on the inhibitory and excitatory postsynaptic potentials (IPSPs and EPSPs) that result in either the hyperpolarization or depolarization of the cell membrane in the dendritic tree (Figure 1A). These stimuli sum up and integrate to generate an action potential in an all-or-nothing fashion. The threshold potential ranges from -55 up to -50 mV depending on the neuronal type (Figure 1B). Differences among action potentials result from the specific set of ion channel complexes found in every neuronal cell type (Figure 1C-D). For instance, Purkinje cells are autorhythmic neurons and contain an ion channel repertoire which allows the cell to repeatedly fire even in the absence of external stimuli, whereas granule cells fire only when stimulated due to the absence of ion channels which can sustain repetitive firing (D'Angelo et al., 2016, 2001).

1.1 *The A-type potassium current*

Different ion channels are responsible for the generation, amplification, and propagation of the postsynaptic potentials and they characterize the electrical makeup of every neuronal cell. Among these ion channels, is the Kv4 channel complex, which is essential for the proper functioning of the heart and the brain. Kv4.3 generates a fast transient outward potassium current, known as the cardiac transient outward current (I_{to}) in cardiomyocytes and the somatodendritic subthreshold A-type K^+ current (I_{SA}) in neurons (Dilks, Ling, Cockett, Sokol, & Numann, 1999; Serôdio, Kentros, & Rudy, 1994; Serôdio, Vega-Saenz de Miera, & Rudy, 1996). In cardiac myocytes, the I_{to} plays a role in the early repolarization of the cardiac action potential (Bohnen, Iyer, Sampson, & Kass, 2015). In neurons, the I_{SA} plays a role in the integration of the postsynaptic signal by dampening the incoming electrical stimulus and determining spike latency (Ramakers & Storm, 2002; Schoppa & Westbrook, 1999; Shibata et al., 2000; Truchet et al., 2012).

Figure 1 Action potential in neurons, and ion channel type, distribution, and gating in Purkinje cells. (A) Schematic representation of a neuron (brown) which receives an excitatory (green neuron) and inhibitory (red neuron) input which generate an excitatory post synaptic potentials (EPSPs) and an inhibitory postsynaptic potentials (IPSPs), respectively. The summation of EPSPs and IPSPs results in the generation of an action potential at the axon hillock. (B) Membrane potential changes for a typical neuronal cell. First, the cell is at rest (V_{rest}), followed by depolarization events which do not reach the threshold potential ($V_{threshold}$), eventually the threshold is reached and the action potential is fired. In the end, the membrane potential hyperpolarizes (V_{AHP}). (C) Left, schematic depiction of a Purkinje neuron. Right, ion channel type and distribution are shown for eight electronic sections (i.e., dendrites with three different diameters, soma, action initial segment (AIS), paraAIS, Ranvier nodes, and collateral) of a Purkinje cell model, based on immunohistochemical data. (D) Representative steady-state activation (solid line) and inactivation (dash line) curves are shown for few selected channels. Adapted from (Masoli, Solinas, & D'Angelo, 2015).

2 The cerebellum

The brain is the central organ of the central nervous system (CNS) and allows us to move, behave, and sense the world around us. Neurons and non-neuronal cells are the basic building blocks of the brain. The former ones are responsible for moving and integrating the information along the neuronal network in the form of electrochemical signals, while the latter ones provide the necessary support for propagating this signal and maintaining brain homeostasis. Different types of neuronal cells are located in different parts of the brain and organize in highly specialized structures to carry out specific functions.

Figure 2 Overview of the cerebellum. (A) The cerebellum (coronal view) is divided into three regions, namely spino-, cerebro-, and vestibulocerebellum with respect to the origin of input. (B) Connectivity of the cerebellum to the rest of the brain. The cerebellum (blue) receives a copy of the motor cortex (turquoise) output via the pontine nuclei (ochre). Based on this, the cerebellum predicts a sensorial response which is then compared to the actual sensory feedback. If there is a mismatch between the two, the cerebellum sends a corrective signal which directly modulates the movement or the motor plan (red arrows). (C) General organization of the cerebellum. Multizonal microcomplexes are formed by several non-adjacent microzones located within the cerebellar cortex. These microzones are made of different neuronal cell types which are highly organized in a three-dimensional structure. The mossy fibers (ochre) are input coming from outside the cerebellum and contact the Granule cells (dark green) whose firing activity is modulated from Golgi cells (cyan). Purkinje cells (dark blue) receive two inputs from mossy and climbing fibers (red), and are inhibited from the molecular layer interneurons, namely stellate (magenta) and basket (light green) cells (see text for more details).

The cerebellum, which means literally “little brain” in Latin, is a structure located in the hindbrain and is divided in three areas depending on the

differences in the sources of input (Figure 2B). The cerebrocerebellar area is the largest in humans and it receives input from several cerebral cortex areas through the anterior pontine nuclei. The other two areas are the vestibulocerebellar and spinocerebellar one. These receive input from the vestibular nuclei in the brainstem (including vestibular nuclei and the inferior olive (IO)) and from the spinal cord through spinocerebellar tracts, respectively. The only output of the cerebellum is through the deep cerebellar nuclei (DCN), which then project to brainstem nuclei and the cerebral cortex through the thalamus, with the exception of the vestibulocerebellum that projects directly to the vestibular nuclei.

The cerebellum indirectly modulates several motor and cognitive functions. The cerebrocerebellum primarily connects to the cerebral cortex and it participates in the coordination of voluntary movement and the performance of cognitive tasks. The vestibulocerebellum connects to the vestibular system and is involved in the coordination of body balance and eye movement. The spinocerebellum connects to both the sensorimotor systems and the cerebral cortex, and is involved in the maintenance of gait (D'Angelo, 2018; Purves D, Augustine GJ, Fitzpatrick D, 2001) (Figure 2A).

2.1 *The cerebellar circuitry*

Although the cerebellum connects to different parts of the brain to regulate a variety of body functions, it is formed by the same repetitive unit: the cerebellar microzone (Andersson & Oscarsson, 1978). Several non-adjacent microzones which connect to the same group of DCN and IO form a multizonal microcomplex (Apps & Garwicz, 2005). These microcomplexes are connected to different extracerebellar structures whose function is modulated by the cerebellum. Each microzone constitutes the most elementary functional unit inside the cerebellum and consists of a specific set of neuronal cells organized into three layers: the granular, Purkinje cell, and molecular layer (going from innermost to outermost) (Figure 2C). In the granular layer, we find the excitatory granule cells (GrCs) and the inhibitory Golgi cells (GoCs) (Golgi, 1885). These are activated from the mossy fibers (MFs), which are axons coming from outside the cerebellum (Eccles, Ito, & Szentágothai, 1967). Other cells are also found in the granular layer; these are the inhibitory Lugaro cells and excitatory unipolar brush cells (UBCs), mainly found in the flocculonodular lobe (Lugaro, 1894; Mugnaini, Sekerková, & Martina, 2011). In the Purkinje cell layer, we find the cell body of Purkinje cells (PCs) whose dendritic tree and axon are situated in the molecular and granular layer, respectively. In the molecular layer, we find the molecular

layer interneurons (MLIs: stellate and basket cells), the dendritic tree of GoCs, and the parallel fibers which are formed by the bifurcated axon emerging from the GrCs. Every parallel fiber runs on a plane, which is perpendicular to the sagittal plane, where the PC dendritic tree and the molecular layer interneurons lie. One parallel fiber contacts several hundred PCs, but has a few synapses on each individual one. The dendritic tree of PCs is also innervated by climbing fibers (CFs), which are axons originating from the IO and activate 5 to 10 PCs. Every PC is innervated from an individual CF and over 100.000 parallel fibers, and inhibits DCN which are activated by the collaterals of the two cerebellar inputs, MFs and CFs. Alongside neuronal cells we also find glial cells (*i.e.*, oligodendrocytes, NG2 cells, microglia, astrocytes, and Bergmann cells) that have different roles, such as myelination of axons and restriction of neurotransmitter diffusion (Apps & Garwicz, 2005; D'Angelo, 2018; Manto & Molinari, 2016; Streng, Popa, & Ebner, 2018).

The neurons forming the cerebellar circuit have specific physiological properties. These properties define how the cerebellum performs its regulatory function. The activity of GrCs, which are silent at rest, is regulated by the competing excitatory and inhibitory input coming from MF and GoC axonal terminals that terminate onto the dendritic tree of GrCs to form a specialized structure, known as the cerebellar glomerulus. When excited, GrCs fire with a frequency up to 300 Hz and transduce the incoming bursts from MFs. These bursts travel to the cerebellar cortex via the PF and modulate the autorhythmic activity of GoCs, PCs and MLIs. While PF input generates simple spikes with a frequency between 50 and 125 Hz in PCs, CF discharge results in complex spikes generation with a frequency around 1 Hz. Thus, PF and CF modulate the firing pattern of PCs. The firing pattern of PCs is also modulated by MLIs, which inhibit PCs at the level of the soma (basket cells) and dendrite (stellate cells). The PC dendritic tree integrates these incoming signals to fine tune the firing pattern of DCN, which eventually determines the performance of motor and cognitive tasks. In order to properly perform its function, the cerebellar circuit presents different levels of plasticity (*e.g.*, GrC-MF, PF-PC, CF-PC) in the form of long-term potentiation and depression. This synaptic plasticity constitutes the molecular basis of motor and cognitive learning (D'Angelo, 2018).

2.2 *Kv4 channel distribution in the brain*

Kv4 channels constitute a family of three channels, namely Kv4.1, Kv4.2, and Kv4.3, which are differently distributed throughout the brain, such as the hippocampus and the cerebellum. Kv4.2 and Kv4.3 mRNA is

abundant in adult rat brain, while Kv4.1 is virtually absent (Serôdio et al., 1994, 1996; Serôdio & Rudy, 1998). Kv4.2 and Kv4.3 channel proteins have been detected both in rat and mouse cerebellum (Amarillo et al., 2008; Otsu et al., 2014), and the A-type potassium current which is mediated by Kv4 channels has been recorded in mouse and rat cerebellar Purkinje cells (Sacco & Tempia, 2002; Y. Wang, Strahlendorf, & Strahlendorf, 1991). The cellular localization of Kv4.2 and Kv4.3 varies across different cerebellar regions. In mouse brain, Kv4.2 and Kv4.3 are highly expressed in the granule cell layer and in the molecular layer, respectively (Amarillo et al., 2008). Kv4.2 protein is found in granule cells within the dendritic region and in the proximity of the soma, while Kv4.3 protein is expressed in the dendrites of mouse Purkinje cells. A similar localization pattern is observed in rat cerebellum both for Kv4.2 and Kv4.3 proteins (Strassle, Menegola, Rhodes, & Trimmer, 2005). One study reports that the Kv4.3 is expressed in granule cell, Lugaro cells, basket cells, and stellate cells, but not in Purkinje cells in rat cerebellum (Hsu, Huang, & Tsaur, 2003). On the contrary, a different study reports that the Kv4.3 protein is found in Purkinje cells dendrites (Otsu et al., 2014). In the human brain, the mRNA which encodes for the Kv4.1, Kv4.2, and Kv4.3 proteins has been detected using RT-PCR analysis (Dilks et al., 1999). While the *KCND1* mRNA transcript is expressed throughout the brain, the *KCND2* and *KCND3* mRNA were only found in the cerebellar grey matter, suggesting that the Kv4.2 and Kv4.3 channel may only be expressed in the dendrites of Purkinje, Golgi cells, and MLIs (Isbrandt et al., 2000).

2.3 *KChIPs and DPLP distribution in the brain*

Two types of auxiliary subunits associate with the Kv4 channel *in vivo* and shape the fast-transient potassium current; these are Kv channel-interacting proteins (KChIPs) and dipeptidyl peptidase-like proteins (DPLPs), whose mRNA splice variants are expressed in different brain regions. While, in rat, mRNA encoding for KChIP2c and KChIP4c are totally absent in the brain, mRNA encoding for KChIP1, KChIP2a/b, KChIP3, and KChIP4a/b/d/e are expressed throughout the brain, in human and mouse (Pruunsild & Timmusk, 2005). At the protein level, KChIP1 and KChIP3 are found in the cerebellum mainly in the granule layer, but not homogeneously in all regions. On the contrary, KChIP4 is homogeneously expressed throughout the cerebellum, whereas KChIP2 is not expressed at high level when compared to other regions in the brain (Strassle et al., 2005). In rat brain, DPLP10a mRNA is found in the cortex, while DPLP10c/d mRNA has been detected in the cerebellum using RT-PCR (Jerng, Lauver, & Pfaffinger, 2007). DPLP expression has been confirmed using immunolabeling for DPLP10, which is mainly expressed

in the Purkinje cell layer, moderately in the granule layer and weakly in the molecular layer (Wang†, Cheng†, & Tsaur, 2015).

3 Kv4.3 channel

Kv4.3 is a voltage-gated potassium channel. In general, potassium channels are transmembrane proteins found in prokaryotic as well as eukaryotic organisms that, upon activation, allow the passage of potassium ions through the cell membrane (Booth, Miller, Müller, & Lehtovirta-Morley, 2014; Doyle et al., 1998). In humans, potassium channels have different physiological roles such as muscle contraction, cell volume regulation, neurotransmitter release, and heart beat rate regulation. These channels are triggered to open by various stimuli including binding of ligands and changes in voltage or pressure.

In mammals, there are 70 known genes coding for potassium channels. Based on their secondary structure, these potassium channels have been classified in four families: (i) two transmembrane, (ii) four transmembrane, (iii) six transmembrane and (iv) seven transmembrane segments (González et al., 2012). The six transmembrane segment family includes the subfamily of voltage-gated potassium channel Kv4 (Figure 3A).

In humans, the Kv4 channel family, also known as the *Shal*-type, is composed of three members: Kv4.1, Kv4.2, and Kv4.3. Kv4.3 has also two isoforms, a short and a long one, that differ from each other at their C-terminal ends (Isbrandt et al., 2000). Kv4 channels share similar structural features. As can be seen in Figure 3B, the channel has a voltage-sensing domain (VSD), a S4-S5 linker, a pore domain, and two cytoplasmic domains, at the N- and C-terminus, respectively. The VSD senses voltage changes across the cell membrane and triggers the opening of the channel pore via the S4-S5 linker, while the N-terminal domain is essential for the tetramerization and cellular localization of the channel, and together with C-terminal domain modulate the activity of the channel (Barghaan & Bähring, 2009; M. Li, Jan, & Jan, 1992; Patel & Campbell, 2005).

Figure 3 Potassium channels overview based on primary sequence similarities. (A) Potassium channels grouped based on primary sequence alignment. (B) Sequence alignment of transmembrane domains from bacterial (P0A334), fruit fly (P08510), human Kv1 (P16389), Kv2 (Q14721), Kv3 (Q96PR1), and Kv4 (Q9UK17-2) channels. α -helical domains (spiral black line), voltage-sensing domain (red), S4-S5 linker (green), and pore domain (blue) are shown. The alignment has been generated using ClustalOMEGA and Clustal coloring, and numbers refer to human Kv4.3.

3.1 *The voltage-sensing domain*

In voltage-gated potassium channels (Kvs), VSD is responsible for sensing changes in the cell membrane potential. This domain is constituted by the first 4 transmembrane segments (*i.e.*, S1-S2-S3-S4). The VSD is a modular functional unit that is found not only in Kvs, but also in other voltage-regulated proteins, such as the voltage-sensing phosphatase (CiVSP), an enzyme whose function is regulated by voltage across the cell membrane (Murata, Iwasaki, Sasaki, Inaba, & Okamura, 2005). Several amino acids (*e.g.*, arginine, lysine, aspartic acid, glutamic acid) are key to sense voltage changes and are highly conserved in VSD throughout evolution (Palovcak, Delemotte, Klein, & Carnevale, 2014). As seen in Figure 3B, the most important ones are the arginine and lysine in the S4 segment. In Kvs, these positively charged residues are found at every third position spaced by two hydrophobic amino acids. The number of arginine/lysine varies from 7 in Kv1.2 (*i.e.*, **RVIRLVRFRIFKLSRHSK**) to 5 in Kv4.3 (*i.e.*, **RVFRVFRIFKFSR**). These amino acids carry charges that build up electrostatic energy in relation to their position within the membrane electric field. This energy can be harnessed to drive conformational changes, which ultimately lead to the opening of the channel (Aggarwal & MacKinnon, 1996; Ishida, Rangel-Yescas, Carrasco-Zanini, & Islas, 2015; Seoh, Sigg, Papazian, & Bezanilla, 1996). The role of charged amino acids has also been investigated for the Kv4.3 channel. It is known that the arginine residues in the S4 segment are crucial for determining the voltage threshold of activation. These arginine residues are also involved in inactivation, closed-state inactivation and recovery from inactivation (Skerritt & Campbell, 2007, 2008, 2009a). Moreover, other charged residues are also essential for the generation of ionic current; these are located in the S2 (*i.e.*, E240) and S3 (*i.e.*, D263) transmembrane helices (Skerritt & Campbell, 2009b).

3.2 *The S4-S5 linker and the pore domain*

The VSD is essential for the proper functioning of Kv4 channels, but the channel could not conduct any potassium ions without the presence of the S4-S5 linker and pore domain, which are essential for physically coupling the movement of the VSD to the pore domain and for forming an energetically favorable path for the passage of potassium ions, respectively.

Figure 4 Kv4 channel model. (A, B) A model of the Kv4.3 channel (excluding cytoplasmic domains) was generated by our collaborators (Giuseppe Brancato et al.). The side and top view are shown on the left and right. We have highlighted the voltage-sensing domain (VSD), the S4-S5 linker, the pore domain and the selectivity filter in red, dark green, and blue.

As can be seen in Figure 4A, the S4-S5 linker (in green) runs parallel to the intracellular membrane surface, and it interacts with the S5 and S6 helix bundle (in blue). When all four linkers are independently activated, they trigger the concerted movement of the S5 and S6 helix bundle, which moves outwards and opens the pore, as shown in the structure of the Kv1.2 channel (Long, Campbell, & Mackinnon, 2005).

In Kv4 channels, the S5 and S6 helices form the pore domain which contains two functional components, namely the selectivity filter and the internal gate, also known as A-gate. The former one is essential for the selection of potassium over sodium ions (hundreds of times more likely) and it is encoded by a highly-conserved amino acid sequence (*i.e.*, TVGYG) found across all Kv channels (Heginbotham, Lu, Abramson, & MacKinnon, 1994). Its selectivity is given by the precise spacing of oxygen atoms within the pore cavity; this molecular funnel favors the replacement of the hydration shell for potassium, but not for sodium ions (Bezaniilla, 2004) (Figure 4A,B).

The internal gate, as the selectivity filter, constitutes a physical barrier, but is not selective; this gate needs to be open for potassium ions to flow through, as shown in experiments, where pore blockers could bind to the channel pore from the inner side, only when the channel was open (Armstrong, 1969; Fineberg, Szanto, Panyi, & Covarrubias, 2016). The

internal gate is formed by the S5 and S6 helices in the form of a four-blade diaphragm of a camera (Figure 4B). In Kv4 channels, the opening and closing of this cavity is controlled by interaction between the S4-S5 linker domain and the S6 helix. Specifically, in the Kv4.2 channel, E323 and S322, found within the S4-S5 linker, interact with the V404 and N408 located in the S6 helix and are important for channel function, as shown by double mutant analysis. The interaction between E323 and I412, not predicted from inspection of available crystal structure, has also been reported. Moreover, there is evidence that interaction between the S4-S5 linker and the S6 helix, within the same subunit and between neighboring ones, is also important for the functioning of the Kv4 channel (Barghaan & Bähring, 2009; Wollberg & Bähring, 2016).

3.3 *The N- and C-terminal domains*

The Kv4 channels, as well as other Kv channel, have two cytoplasmic domains at the N- and C-terminus, respectively. The N-terminal domain plays a role in tetramerization, cellular localization, and inactivation of the channel. First, the channel tetramerization is mediated from specific amino acids (*i.e.*, F87, F110, I121, Y125, F148, Y149; given for Kv1.1) found in the T-domain and conserved across all Kv channels (Strang, Cushman, DeRubeis, Peterson, & Pfaffinger, 2001). Several amino acids (*i.e.*, H104, C110, C131, and C132) are necessary for the coordination of a zinc ion and, when mutations are introduced at these positions, the Kv4 channel is trapped in the endoplasmic reticulum (ER) and found as a monomer (Kunjilwar, Strang, DeRubeis, & Pfaffinger, 2004; G. Wang et al., 2005). Second, the N-terminus contains an endoplasmic reticulum retention sequence, which drives the channel localization towards the ER and away from the plasma membrane. This sequence can be masked by KChIP and results in an increased surface expression for the Kv4 channel (Bähring, Dannenberg, et al., 2001; Pioletti, Findeisen, Hura, & Minor, 2006). Third, the N-terminal domain regulates the channel activity by speeding up inactivation kinetics, although this type of inactivation is prominent in most Kv channels, but not in Kv4 (Jerng & Covarrubias, 1997).

The C-terminal domain is also involved in the regulation of channel gating as well as in the modulation of Kv4 channel activity by KChIP. In the case of Kv4.1, short deletion (up to 96 amino acids) of the C-terminus had no effect on channel activity, but long deletions (from 158 up to 230 amino acids) resulted in the loss of fast inactivation, similar to N-terminal deletion (Jerng & Covarrubias, 1997). In another study, the interaction between the Kv4.2 channel and KChIP2 is affected by deletion within the

C-terminal region (Callsen et al., 2005a). Furthermore, phosphorylation sites, found within the C-terminus, have effect on Kv4 channel activity, as was shown for the Kv4.3 channel, where phosphorylation of T504 affects close-state inactivation kinetics (Xie, Bondarenko, Morales, & Strauss, 2009).

3.4 The Kv4 channel working mechanism

In general, Kv channels open upon depolarization of the cell membrane; the VSD physically moves to an activated position and drags the S4/S5 linker outwards pulling the S5-S6 helices with it, as an opening blade of the camera's diaphragm. The channel inactivates via two pathways: open-state inactivation (OSI) and closed-state inactivation (CSI). Figure 5 illustrates the current gating model of Kvs which are governed from different mechanism of activation and inactivation.

In the case of OSI, on one hand, the channel inactivates from its open state, as the name suggests, and the inactivation takes place via two mechanisms. These two mechanisms were historically named N-terminal and P/C-type inactivation given the involvement of the N-terminal and C-terminal region of the channel, respectively. In the first case, the N-terminal domain blocks the entrance of the open pore from its cytoplasmic side, thus blocking the ions from reaching the channel pore. In the second case, the channel pore rearranges and results in a non-conducting pore (Bähring & Covarrubias, 2011).

Figure 5 Kv channel working mechanism. (A) Schematic kinetics model showing three possible working mechanism for Kv channels. (B) Top, working mechanism depiction are shown for voltage-gated potassium channel, such as Shaker and Kv1, where the channel inactivates following a P/C-type mechanism both from an open and closed state. Bottom, Kv4 channel follows a different mechanism, recently named A/C-type, where the channel enters an inactive state after the VSD loses contact with the pore domain.

In the case of CSI, on the other hand, it is well established that N-terminal and P/C-type inactivation mechanisms play a minor role in the inactivation of the Kv4 channel compared to other Kv channels (e.g., Shaker, Kv1). Indeed, when the N-terminal peptide was either added or deleted, Kv4 current decay was barely affected compared to Shaker channel, while there was no effect on the recovery from inactivation. Furthermore, addition of KChIPs, which eliminate N-type inactivation from the open state by sequestering the N-terminal domain and preventing it from blocking the channel pore, does not significantly affect Kv4 inactivation (An et al., 2000; Barghaan, Tozakidou, Ehmke, & Bähring, 2008; Callsen et al., 2005b; Gebauer et al., 2004; Jerng & Covarrubias, 1997). Another piece of evidence which supports the absence of OSI, mediated by a P/C-type mechanism, in Kv4 channels, is that external tetraethylammonium (TEA), which binds to the exit of the pore in Shaker channel and modulates its activity, does not have any effect on Kv4 channel inactivation and high external potassium concentration accelerate inactivation for Kv4 instead of slowing it down, as shown for Shaker (Jerng & Covarrubias, 1997; Kaulin, De Santiago-Castillo, Rocha, & Covarrubias, 2008; López-Barneo, Hoshi, Heinemann, & Aldrich, 1993; Shahidullah & Covarrubias, 2003). Although, in the absence of external potassium, Kv4 channels inactivate quicker than in the presence of it, suggesting a P/C-type mechanism (Eghbali, Olcese, Zarei, Toro, & Stefani, 2002). Last, the residue which, if mutated from threonine (449) to valine, abolishes P/C-type inactivation in Shaker, is already occupied by a valine in Kv4 channels (López-Barneo et al., 1993). It has been concluded that Kv4 channels inactivate following a CSI pathway via an inactivation mechanism, which has been named A/C-type to highlight the role of the A-gate in contrast to the P-gate, located in the selectivity filter. Other Kv channels also inactivate via a CSI pathway, as in the case of Kv1.5, but they follow a P/C-type mechanism which may coexist along with an A/C-type one (Bähring, Barghaan, Westermeier, & Wollberg, 2012; Kurata, Doerksen, Eldstrom, Rezazadeh, & Fedida, 2005).

Although the CSI mechanism is not fully understood, the available evidence shows that CSI takes place when the channel is in its closed state following an A/C-type inactivation mechanism (Bähring et al., 2012). This involves the desensitization of the VSD that results in the closure of the internal gate, namely the A-gate. The involvement of the VSD in CSI has been proven by double-mutant cycle analysis, where pairs of amino acids, located in the VSD and pore domain, have been shown to affect Kv4 channel inactivation (Barghaan & Bähring, 2009; Wollberg & Bähring, 2016). More evidence for inactivation mediated via an A/C-type

mechanism comes from a study, where the inactivation rate of the Kv4 channel correlates with the rate of VSD desensitization to voltage (Dougherty, De Santiago-Castillo, & Covarrubias, 2008). Last, there is evidence that the internal gate closes upon inactivation of Kv4 channel in good agreement with CSI (Fineberg et al., 2016). Overall, although the CSI mechanism is not fully understood, it dominates the working mechanism of the Kv4 channel via an A/C-type mechanism, although some vestigial form of N-type and P/C-type inactivation may still exist.

3.5 *Regulation of Kv4 channel activity from auxiliary (KChIP and DPLP), accessory, and other cytosolic proteins*

In vivo, the Kv4 channel interacts with two types of auxiliary proteins, namely KChIPs and DPLPs, one type of accessory protein (Kv β) and several protein kinases. KChIP is a cytoplasmic protein, which binds to the N-terminal domain of Kv4, while DPLP is a membrane protein putatively interacting with the VSD via its single transmembrane domain (Strop, Bankovich, Hansen, Christopher Garcia, & Brunger, 2004; H. Wang et al., 2007a). Together, these regulatory proteins modulate the Kv4 channel activity, while accessory proteins affect the expression pattern of the Kv4 channel, auxiliary proteins and kinases regulate the expression pattern, but also channel function (Kitazawa, Kubo, & Nakajo, 2014, 2015; Ren, Hayashi, Yoshimura, & Takimoto, 2005; Strop et al., 2004; H. Wang et al., 2007b).

3.6 *Kv channel-interacting proteins*

KChIP has four members (KChIP1, KChIP2, KChIP3, and KChIP4) with several splice variants. These 200 – 250 amino acids long proteins belong to the neuronal calcium sensor (NCS) family. Some KChIPs possess sequences that favor their association to the lipid membrane via their N-terminal region; otherwise, they are found in the cytosol (Pruunsild & Timmusk, 2005). In KChIP1 and KChIP2-4, there is a myristylation and palmytoilation sequence, respectively (O'Callaghan, 2003; Takimoto, Yang, & Conforti, 2002). The core region of KChIPs contains four putative calcium/magnesium-binding domains called EF-hand motif (EF) (*i.e.*, EF1, EF2, EF3, and EF4). EF1 is degenerated and does not bind any ions, EF2 binds magnesium and EF3-4 have high affinity to calcium. Overall, these EF motifs make KChIP a highly sensitive calcium sensor that can rapidly respond to changes in the intracellular concentration of this bivalent cation (Bähring, 2018). Given its ability to sense calcium, KChIP has been attributed several roles. Among these, are the formation and trafficking of the Kv4-KChIP complex, and the gating of the Kv4 channels.

Conflicting results have been reported regarding the role of calcium in the formation of the Kv4-KChIP channel complex. The formation of the Kv4.3-KChIP1 has been shown to be calcium-dependent, as in the case of Kv4.2-KChIP4b1 and Kv4.3-KChIP3 (Gonzalez, Pham, & Miksovska, 2014; Morohashi et al., 2002; Pioletti et al., 2006). On the contrary, Kv4.2-KChIP1 and Kv4.2-KChIP2 complexes do not require the presence of calcium for their formation (An et al., 2000; Bähring, Dannenberg, et al., 2001). Although further experiments are necessary to elucidate the role of calcium in the formation of the channel complex, differences among KChIPs and Kv4 channels may account for this discrepancy. Furthermore, it is known that calcium is necessary for the trafficking of Kv4.2-KChIP1 complex to the plasma membrane, while nothing is known about the effect of calcium on the Kv4:KChIP stoichiometry (Hasdemir, Fitzgerald, Prior, Tepikin, & Burgoyne, 2005). Moreover, KChIP1 interaction with the N-terminal of Kv4.3 has been documented from two independent studies, where the crystal structure of the N-terminally bound KChIP1 was solved (Pioletti et al., 2006; H. Wang et al., 2007b). These studies show that one KChIP interacts at two sites on the channel: the N-terminal and tetramerization domain, located on two adjacent subunits. An interesting idea is that calcium may differentially affect the binding of KChIP to these sites, supported by electrophysiological recording in the presence of strong calcium buffering (Groen & Bähring, 2017).

Upon binding to Kv4, KChIPs modulate the surface expression and the activity of the Kv4 channel. There is plenty of evidence that the expression of KChIPs, with the exception of KChIP4a, increase the surface expression of Kv4 channels by masking an endoplasmic reticulum retention sequence, located on the N-terminal region of the Kv4 channel (Bähring, Dannenberg, et al., 2001). In the case of KChIP4a, a K⁺ channel inactivation suppressor (KIS) sequence in its N-terminal region is responsible for the retention of Kv4 channel within the endoplasmic reticulum (Tang et al., 2013).

KChIP also modulates the gating properties of the Kv4 channel by modulating the activation and inactivation kinetics, and the recovery from inactivation. For instance, KChIP2.2 abolishes the fast phase of inactivation, known as N-type inactivation, by trapping the N-terminal domain of the Kv4.2 channel, as shown in the crystal structure of the KChIP-Kv4 complex (Bähring, Boland, Varghese, Gebauer, & Pongs, 2001; Pioletti et al., 2006). However, N-type inactivation does not play a prominent role in Kv4 channels. Another effect of KChIPs is to accelerate the slow phase of channel inactivation, as shown by the effect of KChIP1

on Kv4.1 and Kv4.3. However, this effect depends on the type of the KChIP. For instance, KChIP4a has the opposite effect on inactivation (Holmqvist et al., 2002). Generally, most KChIPs slow down and speed up the fast and slow phase of inactivation for all Kv4 channels. Moreover, KChIP1 speeds up the recovery from inactivation of the Kv4 channel, respectively (Beck, Bowlby, An, Rhodes, & Covarrubias, 2002). Although this is the case for most KChIPs, KChIP1b has the opposite effect and results in a recovery from inactivation for the Kv4.2 channel (Van Hoorick, Raes, Keyzers, Mayeur, & Snyders, 2003).

3.7 *Dipeptidyl peptidase-like proteins*

The other auxiliary subunit which modulates the cellular localization and the activity of the Kv4 channel is DPLP, also known as DPP. This group of proteins has several members, such as DPLP10, DPLP6, and DPLP4 in different splice variants. DPLPs have three domains: a short cytoplasmic N-terminal, one transmembrane helix and one extracellular domain. Generally, DPLPs increase the surface expression, accelerate the kinetics of inactivation, and shift the ionic current window of the Kv4 channel to more hyperpolarized potentials (Jerng, Qian, & Pfaffinger, 2004). For certain DPLPs, the N-terminus acts as the N-terminal of the Kv4 channel; it plugs into the open pore from the cytoplasmic side and blocks ionic conduction, resulting in the development of fast inactivation. This has been observed for the DPP6a and DPP10a whose N-terminal sequence could be transplanted to DPP6S and resulted in fast inactivation, not observed in the presence of DPP6S alone (Jerng et al., 2007).

Although most DPLPs accelerate the recovery from inactivation of the Kv4 channel, it is not the case for DPP6K. This variant slows down the recovery kinetics and it also shifts the ionic current window to more depolarized membrane potentials. This unique activity has been linked to specific amino acids located in the cytoplasmic N-terminal region (Jerng & Pfaffinger, 2011; Nadal, Amarillo, de Miera, & Rudy, 2006).

3.8 *Accessory proteins and protein kinases*

There are also other regulatory proteins reported to affect the Kv4 channel activity, such as accessory proteins belonging to the Kv β family. They affect the surface expression of the Kv4 channel, as shown in the case of Kv β 2 and Kv β 1, which result in the increase and decrease of the peak current density for the Kv4 channel, respectively (L. Wang, Takimoto, & Levitan, 2003; Yang, Alvira, Levitan, & Takimoto, 2001). In addition, there are also different kinases (*i.e.*, PKA, PKC, and ERK) that modulate the

Kv4 channel activity. Kv4 has several putative phosphorylation sites some of which have been validated to affect the Kv4 channel activity (Adams et al., 2008). For instance, if PKA phosphorylates Kv4.2 within the C-terminal region, the activation of the channel is shifted towards more depolarized membrane potentials (Schrader, Anderson, Mayne, Pfaffinger, & Sweatt, 2002).

To summarize, at the molecular level, the Kv4 channel is responsible for the fast inactivating potassium current, found in different types of electrical cells, such as neurons and cardiomyocytes. In these different cell types, specific subset of regulatory proteins (e.g., KChIPs, DPLPs, and PKA) fine tune the potassium current in two ways. First, the regulatory proteins determine the intensity of the current at the plasma membrane by affecting the surface expression/trafficking of the channel and by accelerating or slowing down the current kinetics. Second, they tune the sensitivity of the Kv4 channel to membrane voltages by shifting the voltage window of its activity and reducing/increasing the availability of active channels. Overall, this results in the generation of an *ad hoc* potassium current, which characterizes the electrical activity of specific cell types throughout our body.

4 Channelopathies

Malfunctioning of ion channels gives rise to several medical conditions, known as channelopathies. These disorders affect different biological systems, such as the cardiovascular, nervous, respiratory, and immune systems (Kim, 2014). In the heart, the synchronous activity of ion channels determines the shape of the cardiac action potential. When ion channels do not properly function, cardiac arrhythmias occur and, in some cases, cause sudden cardiac arrest. Mutations which result in sudden cardiac arrest have been identified in many genes encoding for ion channels (*i.e.*, calcium, sodium, potassium channels) (Amin, Tan, & Wilde, 2010). In the nervous system, whose functioning relies on the activity of ion channels, several neurological disorders are also the result of mutations in genes encoding for ion channels. For instance, the first identified and best-understood neuronal channelopathies include the ones that cause primary skeletal disorders. Patients affected from these disorders show a clinical spectrum ranging from myotonia to flaccid paralysis, and carry mutations in a skeletal muscle chloride channel, CIC-1 (Imbrici et al., 2015). Another example is Dravet syndrome that is a severe form of epilepsy. This specific form results from mutations in a voltage-gated sodium channel gene (*SCN1A*) or in a GABA-activated chloride channel

subunit (GABRG2) (Huang, Tian, Hernandez, Hu, & Macdonald, 2012; Scheffer, Zhang, Jansen, & Dibbens, 2009).

In the case of the Kv4.3 channel, mutations have been linked to heart arrhythmias (e.g., Brugada syndrome) and a neurological disorder (e.g., spinocerebellar ataxia 19/22) (Duarri et al., 2012; Giudicessi et al., 2012; Lee et al., 2012). Interestingly, one single mutation has never been reported to cause both diseases in the same individual (Duarri et al., 2013).

4.1 *Heart arrhythmias*

In 1992, Brugada syndrome (BrS) was reported for the first time and associated with sudden cardiac death (SCD) (Brugada & Brugada, 1992). Currently, BrS accounts for 12% of SCD and 20% of SCD of patients with no structural abnormality in the heart. After the first report, several case studies followed and, in 1998, the first genetic cause was identified in the gene coding for the voltage-gated sodium channel Nav1.5 (Chen et al., 1998). This has led to the classification of BrS as a hereditary heart condition. Although the majority of patients remain asymptomatic, some present ventricular fibrillation, which causes syncope or SCD. Although the molecular mechanism leading to this arrhythmia is not well understood, over the last couple of decades, many pathogenic mutations have been linked to BrS in 19 different genes. Many of these genes encode for a variety of ion channels and regulatory proteins expressed in the heart (Nielsen, Holst, Olesen, & Olesen, 2013). These proteins are responsible for the generation of different ionic currents, which make up the cardiac action potential. Among the affected genes, several ones encode ion channels, such as voltage-gated sodium, calcium, and potassium channels, including the Kv4.3. BrS is not the only type of arrhythmia caused by mutations in ion channels. Atrial fibrillation is another disease that results from mutations in genes encoding for different type of ion channels.

Up to date, several mutations in Kv4.3 have been reported to cause heart arrhythmias (Giudicessi et al., 2012, 2011; Olesen et al., 2013; Takayama et al., 2019; You, Mao, Cai, Li, & Xu, 2015). In the heart, Kv4.3 is known to be the molecular basis of the fast inactivating potassium current (I_{to}) in the ventricular epicardium (Dixon et al., 1996; Nerbonne & Kass, 2005). Here, the Kv4 current is responsible for the repolarization of the membrane potential after the action potential has reached its peak. Heart arrhythmias-causing mutations are gain-of-function mutations and increase

the intensity of I_{to} in the myocytes, ultimately accelerating the repolarization of the cell membrane (Giudicessi et al., 2011).

4.2 *Spinocerebellar ataxia 19/22*

The spinocerebellar ataxias (SCAs) are a group of neurodegenerative disorders, inherited in an autosomal dominant way. Patients present a broad variety of symptoms, such as gait and eye movement abnormalities, hearing loss, and poor balance. Mutations in more than 30 genes lead to the development of SCAs (Klockgether, Mariotti, & Paulson, 2019). Over the last decades, several inherited mutations in the coding region of the *KCND3* gene, which encodes for Kv4.3, have been reported to cause SCA19/22 (Duarri et al., 2012; Lee et al., 2012). Moreover, two *de novo* mutations have been identified within the *KCND3* gene in patients that lead to a complex neurological phenotype including early onset cerebellar ataxia (Kurihara et al., 2018; Smets et al., 2015).

The SCA19/22 mutations, reported so far, are loss-of-function mutations. These mutations result in the reduction of I_{SA} current density (Duarri et al., 2012). On one hand, it is known that mutations cause retention of the Kv4.3 channel protein within the endoplasmic reticulum due to protein misfolding. The ER retention leads to protein instability, that can be rescued by the presence of 1) KChIP and 2) the wild-type Kv4 channel (Duarri et al., 2015). On the other hand, the effect of the mutations on the channel function has not been extensively investigated, especially the effect of mutations on the modulation of channel activity from auxiliary proteins. One study has been conducted to investigate the effect of certain SCA19/22 mutations on the channel function in the presence of KChIP2b, but it remains an open question, whether pathogenic mutations have any effect on single-channel conductance (Duarri et al., 2015). Moreover, how SCA19/22 mutations affect the function of the Kv4.3-KChIP complex, specifically relevant in physiological conditions, has not yet been investigated.

5 The structural determinant of ion channel functioning

In order to sense external stimuli and to allow the passage of potassium ions, ion channels are equipped with different structural domains, such as the VSD, SF, and A-gate, as mentioned earlier in this chapter. The three-dimensional arrangement of these domains has always been the object of investigation because it reveals the molecular mechanisms that are responsible for the functioning of ion channels. Currently, two main techniques, namely X-ray crystallography and cryo-EM, are employed to investigate the three-dimensional structure of ion channels. Although

these approaches have generated invaluable amount of information, they also present some limitations. Most importantly, structures, which are obtained from crystallography and cryo-EM studies, may not represent the native physiological states, due to the presence of detergents, buffer solutions, and vitreous ice. Moreover, low-resolution structures provide a limited amount of information which may leave some doubts regarding the orientation of certain structural domains. Finally, static structures do not always give information about dynamic mechanisms, which are crucial to protein functioning.

For this purpose, molecular modelling (MM) has been used and has allowed to fill the gaps left behind from experimental approaches. For instance, MM has been exploited for the refinement of low-resolution crystal structures, for checking the stability of resolved structures, and for providing the structure of proteins whose crystal structure has not yet been solved. In the latter case, the model is built using homology modelling techniques (e.g., MODELLER, Schrodinger Prime, and HHPred) on the assumption that structures are more conserved than sequences (Fiser & Šali, 2003; Jacobson et al., 2004; Zimmermann et al., 2018). This assumption is true in the case of membrane proteins as well as soluble ones, where structures, which have a sequence similarity of 30%-40%, only deviate from each other by few angstroms (Forrest, Tang, & Honig, 2006).

Along with these tools, which are limited to the prediction of static conformation, molecular dynamics (MD) simulations have provided a way to study the thermodynamic and kinetic processes. MD simulations have provided an invaluable resource for understanding the molecular mechanism governing the functioning of ion channels (Conti et al., 2016; J. Li et al., 2017). The latest advancements in computing technology and MD algorithms (e.g., NAMD, GROMACS, and OpenMM) have allowed to run simulations within the microsecond range (Eastman et al., 2017; Hess, Kutzner, van der Spoel, & Lindahl, 2008; Phillips et al., 2005). This time resolution permits the observations of several dynamical processes, such as surface side-chain rotations, loop motions, and ion conductance. Unfortunately, the gating dynamics are still out of reach for most researchers who do not have access to specialized supercomputers (e.g., Anton 2 by DE Shaw Research) (Shaw et al., 2014).

5.1 *The contribution of MD simulations to the study of ion channels*

As already mentioned, in potassium channel, the SF is an essential structural component that is responsible for the selection of potassium

ions with a 10.000-fold preference over sodium ions. This highly selective atomic sift is built from the backbone atoms of a highly conserved sequence (*i.e.*, TVGYG) that line the channel pore (Doyle et al., 1998). From crystallography studies, it has been shown that the potassium atoms occupy four binding sites, known as Site₁-Site₄, while MD simulations have helped to unravel the presence of two more sites (*i.e.*, Site₀ and Site_{cav}), which could not be identified using classical experimental techniques (Bernèche & Roux, 2001; Morais-Cabral, Zhou, & MacKinnon, 2001).

While there is agreement over the structure of the SF, how the ions pass through this narrow passageway is still a matter of debate. Initially, a “soft” knock-on mechanism has been proposed, based on experimental and simulation data. In this scenario, two potassium ions occupy two sites (*i.e.*, Site₃ and Site₁) within the SF and are separated from one water molecule, as shown in the crystal structure of the bacterial KcsA channel and corroborated from MD simulations. The potassium ions file is pushed forward, once a potassium ion enters the Site_{cav}, resulting in two potassium ions moving to Site₄ and Site₂ (Åqvist & Luzhkov, 2000; Bernèche & Roux, 2001). Recently, a different mechanism, known as “hard” knock-on has been proposed. In this case, potassium ions are not separated from water molecules, and are pushed forward from the direct electrostatic repulsion generated from the entrance of other potassium ions within the SF (Kopfer et al., 2014). This second mechanism has been supported from one recent crystallographic study, while the first mechanism is in agreement with a different study, which used a combination of spectroscopy and MD techniques (Kratochvil et al., 2016; Sheldrick, 2015). Finally, a recent MD study has concluded that only the “hard” knock-on mechanism can account for the high conduction efficiency and the ion selectivity of potassium channels (Kopec et al., 2018).

Different inactivation mechanisms have been observed for Kv channels. Among these, is C-type inactivation that involves structural rearrangements of the channel pore. As in the case of channel conductance, while studies agree on the involvement of the pore in C-type inactivation, there is not a consensus regarding the molecular mechanism governing C-type inactivation (Hoshi & Armstrong, 2013). On one hand, based on study performed on the KcsA, the SF has been proposed to collapse due to polar interactions behind the SF (Cordero-Morales et al., 2006; Cuello, Jogini, Cortes, & Perozo, 2010). In this case, MD simulations has been essential for identifying amino acids which were

mistakenly thought to contribute to the inactivation process (J. Li et al., 2017). On the other hand, from a study of Shaker channel, it has been shown that C-type inactivation is linked with a dilation of the SF resulting from the VSD pulling outwards on the pore domain (Conti et al., 2016). Whether this is the main mechanism governing C-type inactivation, also in other Kv channels, needs to be further investigated.

Structural biology experiments and MD simulations have aided the understanding of how different structural domains encode ion channel function. However, there is still a lack of structural information regarding many Kv channels, including Kv4, whose tetramerization domain is the only crystallized domain (Pioletti et al., 2006; H. Wang et al., 2007a). Although high structural similarity is expected among voltage-gated potassium channels (Kvs), there is a need for either experimental or *in silico* data. This will pave the way for an investigation of molecular mechanism which govern the Kv4 channel activity and may unravel unique structural features that are not found in other Kv channels.

6 Scope of the thesis

In this thesis, we aimed at better understanding how the Kv4.3 channel complex works at the molecular level, with the ultimate goal to shed more light on the pathophysiology of SCA19/22. We have done this following two main lines of investigation. First, we have used information from patients, namely inherited and *de novo* mutations, and studied the effect of these mutations on channel electrical activity and gating. Second, we have developed new methodologies and tools for investigating the structure-function relationship of the Kv4.3 channel.

In Chapter 2, we chose two mutations (*i.e.*, M373I and S390N), which respectively cause a mild and a severe phenotype in patients. We characterized the effect of these mutations on channel activity in the presence and the absence of KChIP2b using both electrophysiological and *in silico* 3D modelling at the single-channel and single-cell level. We found that these two mutations cause a slight decrease in single-channel conductance, and alter the modulation of the auxiliary subunit KChIP2b. Moreover, using our 3D model, we have unveiled the effect of the M373I and S390N mutations on the pore region and the external gate, respectively. Our results show how SCA19/22 mutations affect the way KChIP2b modulate the Kv4.3 channel activity.

In Chapter 3, we have characterized the effect of *de novo* mutations on Kv4.3 channel activity and function in the presence and absence of KChIP2b using the same methodologies as described in Chapter 2. We

found that these mutations have quite heterogenous effects. On one hand, some *de novo* mutations (*i.e.*, L310P, T366I, and G371R) result in the complete loss of A-type potassium current, a phenotype that could not be rescued by the presence of KChIP2b. Using computational methodologies, we could show the effect that these mutations have on the structure of the Kv4.3 channel. On the other hand, a couple of mutants (*i.e.*, V294F and V399L) produce whole-cell currents, but show altered gating. This study shows how *de novo* mutations reduce the availability of A-type potassium currents at physiological potentials as a result of different effect on channel localization, structure, or function.

In Chapter 4, we were interested in following the structural and functional changes of the Kv4.3 channel in real-time using a site-specific fluorescent probe on the channel backbone. In order to achieve this, we genetically incorporated fluorescent unnatural amino acids to the Kv4.3 channel. Specifically, we have tested the feasibility to introduce the genetically encoded unnatural amino acid ANAP at several amino acid positions. We have found that the channel can be probed with unnatural amino acids depending on the labeling position on the channel. The buried positions are increasingly more difficult to label than positions at the surface. This proof of concept study shows the feasibility and limitations to label the Kv4.3 channel using the fluorescent amino acid ANAP.

In Chapter 5, we have looked at the effect of the chemical nature of a residue in the exit of the outer pore on channel conductance. We have previously shown that the M373I mutation results in dehydration of the pore region and lower single channel conductance of the Kv4.3 channel, possibly due to the hydrophobic nature of the side chain. Here, we have investigated the role of this amino acid in facilitating the passage of ions through the channel. To achieve this, we mutated the methionine to the hydrophilic residue aspartic acid and assessed the effect of this substitution on channel function and structure using electrophysiological recordings and MD simulations.

In Chapter 6, we have established a protocol to study the Kv4.3 channel complex by a bottom-up approach. We used a heterologous expression system for the production of the Kv4.3 channel protein and established a protocol for its production and purification. Once isolated, the Kv4.3 channel was functionally reconstituted into a lipid bilayer made of lipids extracted from the brain. Although the purification and the reconstitution procedures need further optimization, this work lays the foundation for the study of the Kv4.3 channel complex in a highly-controlled environment by

maintaining full control over the addition of single mutations and other regulatory proteins.

In Chapter 7, we have summarized the main findings, and discussed their implications on the current understanding of Kv4.3-related pathology and the working mechanism of the Kv4 channel complex. We propose a common mechanism which may cause neuronal dysfunction due to loss-of-function mutations in the Kv4.3 channel. Moreover, we put forward the idea that KChIP modulates channel activity by interacting with the cytosolic portion of the Kv4.3 channel. Last, we suggest how the protocols, described in Chapter 4 and 6, may be used in the future to perform structure-function studies on the Kv4.3 channel.

7 References

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Chapter 2

How do SCA19/22 mutations affect the function and structure of the wild-type Kv4.3 channel?

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The voltage-gated potassium channel Kv4.3 plays a vital role in shaping the timing, frequency, and backpropagation of electrical signals in the brain and heart by generating fast transient currents at subthreshold membrane potentials in repetitive firing neurons. In recent years, an increasing number of inherited and de novo mutations on Kv4.3 channel have been identified and, in the case of the brain, shown to cause spinocerebellar ataxia type 19/22. However, how these mutations affect the channel function at the molecular level remains to be elucidated. Here, we present the study of a mild and a severe pathogenic single mutant of Kv4.3, namely M373I and S390N, where their effects on the functioning of the ion channel and on the modulation of the channel activity by an auxiliary protein are described in detail. By employing electrophysiology, homology modelling, and molecular dynamics simulations, we could link functional abnormalities to the structural changes induced by each mutation at the molecular level. Our findings show that the pathogenic mutations not only affect the channel structure and function but also interfere with channel modulation by cell-specific interacting partners.

1 Introduction

The Shal-type protein Kv4.3 is a voltage-gated potassium channel which is highly expressed in the brain and heart (Birnbaum et al., 2004). Kv4.3 forms a heteromeric complex with its auxiliary partners, namely the Kv channel interacting proteins (KChIPs) and the dipeptidyl peptidase-like proteins (DPLPs), and generates somatodendritic transient A-type potassium current in the brain (Rhodes et al., 2004; Strop, Bankovich, Hansen, Christopher Garcia, & Brunger, 2004; Sun et al., 2011). In the cerebellar Purkinje cells (PCs), which are the efferent projection neurons of the cerebellar cortex providing its sole electrical output for fine-tuning the voluntary motor and cognitive activities, A-type current activates at a range of membrane potentials that are subthreshold to action potential generation and controls the timing, frequency, and backpropagation of action potentials (Anderson et al., 2010; Bardoni & Belluzzi, 1993; Sacco & Tempia, 2002; Y. Wang, Strahlendorf, & Strahlendorf, 1991). Due to the role of Kv4.3 in membrane excitability and electrical signaling through A-type current, malfunctioning of Kv4.3 channel complex has pathological consequences on the neuronal activity in the brain and heart (Duarri et al., 2012; Giudicessi et al., 2011; Lee et al., 2012; Smets et al., 2015). In 2012, a number of Kv4.3 mutants were identified in large patient cohorts that were associated with the movement disorder spinocerebellar ataxia type 19/22 (SCA19/22) with no detected effects on the heart function (Duarri et al., 2012; Lee et al., 2012). These pathogenic Kv4.3 mutants cause loss of PCs, leading to the neuronal death and cerebellar atrophy with an unknown mechanism. The patients lose control over their walking, speech, body balance, and eye movements. Currently, there is no cure and treatment to reduce the symptoms. Furthermore, while these hereditary mutants show their effect mostly later in life, recently, *de novo* mutations with devastating effects at very early ages also started to emerge, urging for a better understanding of the (mal-)functioning of Kv4.3 channels (Kurihara et al., 2018; Smets et al., 2015).

Kv4.3 is a tetrameric protein with cytoplasmic N- and C-termini (Figure 1A). Each subunit has a transmembrane domain (TMD) comprised of six alpha helices (S1-S6). While S1-S4 form the modulatory and highly conserved voltage-sensing domain (VSD), S5-S6 helices form the innermost structures and line the pore domain (PD) (Figure 1B-C). Voltage-induced conformational changes in the VSD are coupled to the PD via the S4-S5 linker, and potassium ions are coordinated at the selectivity filter (SF) at the outer end of the S5-S6 loop (Figure 1C). Despite sharing the key characteristic structural features of all voltage-

gated potassium (Kv) channels in its core region (S1-S6), Kv4.3 has specific and highly conserved structural aspects that define its different working mechanism (Jerng, Pfaffinger, & Covarrubias, 2004). It activates and, more importantly, inactivates rapidly with inactivation mechanisms that are different from the conventional Shaker N- or P/C type mechanisms. Before opening with depolarizing voltages, the resting channels first undergo into a closed-active state. Then, upon further depolarization, the channels either open up or, more preferentially, turn into a closed-inactive state (Figure 1D) (Bähring & Covarrubias, 2011; Fineberg, Szanto, Panyi, & Covarrubias, 2016; Jerng et al., 2004; Patel & Campbell, 2005). Again, unlike Shaker-type Kv channels, during recovery from inactivation, Kv4 channels do not re-open (Bähring & Covarrubias, 2011). Inactive channels recover rapidly at hyperpolarized membrane potentials (Jerng et al., 2004). However, the mechanistic details of channel gating are not yet fully understood.

Figure 1 Atomic homology model and current gating model of Kv4.3 channel. (A) Side view of the open conformation of the wild-type (WT) Kv4.3 full-length model in its tetrameric form showing transmembrane (TMD) and intracellular (ICD) domains and KCIP1 auxiliary subunits. Black lines indicate the lipid bilayer. Crystallographic potassium, zinc, and calcium ions are shown in magenta, green, and red, respectively.

Kv4.3 and KChIP1 residues are shown in white and yellow, respectively. For clarity, only two of the four KChIP1 auxiliary subunits are presented. (B) Top view of the WT TMD with the location of voltage-sensing domains VSDs (S1-S4 residues 182-307) and the pore domain (PD) (S5/S6 residues 321-402). (C) Side view of the WT PD showing two monomers. The location of the SF (residues 367-372) is indicated by a black arrow. The sites of point mutations in WT Kv4.3 residues M373 and S390 are highlighted in sky blue and red licorice, respectively. (D) The Kv4.3 channel gating mechanism is depicted (only two monomers for clarity). Upon membrane depolarization, the channels in the resting state (R) enters into a closed-active state (CA). From here, the Kv4.3 either opens (open state, O) and subsequently closes by passing through a short-lived CA state, or fails to open and directly falls into its closed inactive state (CI), adapted with permission from (Bähring & Covarrubias, 2011). The S4 helix, S4-S5 linker, and S5/S6 helices are shown in red, blue, and green, respectively.

Previous studies on pathological Kv4.3 mutants focused on the localization of the channel and its activation and inactivation in the presence of the main auxiliary subunit Kv channel interacting protein KChIP2b (Duarri et al., 2012, 2015; Lee et al., 2012). It has been shown that most of the mutants have trouble reaching the cell membrane in mammalian cells, and some display significant changes in the voltage dependence of gating. However, due to the lack of a suitable crystal structure, it is not clear how such pathogenic mutations affect the Kv4.3 structure and, in turn, how these changes alter the gating mechanism, kinetics, and modulation by KChIP2b at the molecular level.

Here, we investigated how two of the hereditary mutations, namely M373I and S390N, affect the structure and function of the Kv4.3 channel, both in the absence and presence of KChIP2b. M373I and S390N are predicted to be in the selectivity filter and S6 helix, respectively. Both amino acid residues are well-conserved among wild-type (WT) Kv4.3 orthologues in various organisms. While M373I causes mild symptoms in SCA19/22 patients, S390N is a very severe mutation with symptoms ranging from saccadic eye movements to hearing deficits and cognitive impairment (Duarri et al., 2012). First, we generated a homology model of the human Kv4.3 (Figure 1A), which is particularly suitable for mapping the mutation sites onto the protein structure and for allowing structure-function studies. Then, we performed a systematic functional study on the electrophysiological activity of the WT and two mutants at the single-cell and single-channel level. Our data demonstrate that, especially in the presence of KChIP2b, M373I is functionally very similar to the WT, except for a slightly lower single-channel conductance and slower recovery from inactivation.

In the case of S390N, the functionality of the channel at all stages of gating are dramatically affected, both in the absence and presence of KChIP2b,

showing a marked preference for the closed-inactive state. Moreover, molecular dynamics (MD) simulations and structural analysis showed that in M373I mutant the hydration properties and rigidity of the selectivity filter were altered. On the other hand, the helical packing and contacts among gating helices in S390N were significantly different from that of the WT, especially weakening the coupling of S6 helix with the S4-S5 linker and thus favoring channel inactivation.

2 Results

2.1 *A molecular model of the Kv4.3 channel and its mutants*

To gain a mechanistic understanding of the main structural and functional differences induced by single-site mutations with respect to the WT, we generated an atomistic model of the Kv4.3 channel in its open state using a combination of X-ray crystal structures and homology modeling techniques, as described in the Methods section. In particular, the Kv4.3 TMD (residues 165-411) reflects closely the typical architecture observed in other Kv channels, where each monomer is equipped with a VSD (S1-S4, residues 182-307) linked to the central pore-forming S5 and S6 helices, the latter embedding the highly conserved ion selectivity filter (SF, residues 367-372) of the channel (Figure 1A-B). From the model, it can be observed that both mutation sites, namely 373 and 390, are within the PD, but in different spatial regions of the channel (Figure 1B-C). Position 373 is located on the H5 loop connecting S6 with S5 immediately above the SF segment. The M373I mutation introduces a more hydrophobic residue in this interfacial region of the protein facing the extracellular environment (Figure 1B-C). On the other hand, position 390 is approximately located in the middle of the S6 helix (residues 382-402) within the pore lumen and below the SF, and it is oriented away from the channel central axis and towards the S5 helix (Figure 1C): the S390N single mutant introduces a bulkier side chain in a tightly packed interfacial region between S6 and S5. Then, our model suggests that M373I might affect the potassium ion conductance and/or channel inactivation via altering the hydration properties and the rigidity of the SF, whereas S390N might have different and more severe consequences for channel function.

Figure 2 The effect of the M373I and S390N mutations on the whole-cell and the single-channel conductance. Macroscopic currents of the WT, M373I, and S390N channels transiently expressed alone (A) or co-expressed (C) with KChIP2b in Chinese hamster ovary (CHO) cells. Whole-cell currents were evoked by 2s step depolarizations from a holding potential of -90 mV to test potentials in 10 mV increments (see inset). (B) Single-channel currents of WT, M373I, and S390N channels were recorded in the cell-attached configuration of patch clamp at +60 mV preceded by a holding potential of -90 mV. (D) Comparison of the whole-cell conductance of WT and mutants in the presence and absence of KChIP2b. Statistical significance was calculated using the Student's t-test (p -value: * < 0.05, *** < 0.00005). WT, M373I, and S390N are marked with red, blue, and green. Empty and filled histogram columns represent the sample without and with KChIP2b, respectively.

2.2 M373I and S390N reduce both cell and single-channel conductance

To investigate the functional consequences of M373I and S390N mutations on the Kv4.3 channel, we expressed each channel in Chinese hamster ovary (CHO) cells and determined the macroscopic current amplitudes using whole-cell patch clamp electrophysiology. Peak currents were elicited by depolarizing the membrane from a holding membrane potential of -90 mV to voltages ranging from -90 to +60 mV in 10 mV increments (Figure 2A, inset). WT and mutant channels showed the characteristic fast activating and fast inactivating current behavior. However, while the macroscopic current of M373I was slightly lower than that of the WT, the current generated by S390N was only 10% of the WT peak current (Figure 2A, D). The main reasons for lower whole-cell conductance could be malfunctioning of the channels and/or a reduced

number of channels at the plasma membrane. Therefore, we measured the single-channel conductance of each channel by using cell-attached patch electrophysiology in CHO cells. While the single-channel conductance of WT was 11.1 ± 2.7 pS ($n = 5$), that of M373I and S390N were 9.5 ± 2.4 pS ($n = 4$) and 9.5 ± 1.2 pS ($n = 5$), respectively (Figure 2B and Table 1). Therefore, even though conducting slightly less than the WT, M373I and S390N are functional. Based on their single-channel conductance, they could have generated about 80% of the WT conductance, in place of 64 and 10%, respectively. Thus, to test any inefficiency in trafficking of these mutants to the membrane, as reported in other heterologous expression systems, we expressed them together with KChIP2b (Duarri et al., 2012). Indeed, when we co-expressed with this auxiliary protein, the surface expression and the total cell conductance of each channel increased significantly (Figure 2C, D), while the single-channel conductance stayed the same (Table 1). M373I and S390N reached 87% and 48% of the WT cell conductance, respectively, (Figure 2D), indicating more severe effects of S390N on the channel structure-function.

2.3 The effects of M373I and S390N on K⁺ translocation

To shed some light on the molecular determinants causing the observed decrease in single-channel conductance, we carried out a comparative *in silico* investigation between these mutants and the WT Kv4.3 channel. In the following, we present the main results highlighting the structural and functional differences as issuing from one microsecond MD simulations. For both WT and mutants, Kv4.3 TMD was embedded in a POPC lipid bilayer and exposed to a 0.5 M KCl aqueous solution, while an applied voltage of 1 V was maintained throughout the simulation in order to study the channel during its active state at exercise conditions. In the case of M373I, we noticed only minor changes in the protein average structure with respect to WT, as shown by the alignment of the equilibrated structures of both systems (Figure 3A, inset), the very similar residue-residue contact map (Figure S1), and the relatively small root-mean-square deviations (RMSD < 2 Å) with respect to the starting WT reference structure (Figure S2). Moreover, the average pore size measured along the channel longitudinal axis also showed no significant differences: both channel forms displayed a pore radius of about 4 Å in the so-called cavity region of the TMD, which immediately precedes the narrow SF stretch where the pore radius drops to 1 Å (Figure 3A). Despite such similarities in global protein structure, the estimated ion conductance, which was evaluated from the observed K⁺ permeation events, showed a noticeable decrease in M373I, about 40% less (Table

S1). From the analysis of the contacts formed by residue 373 with other PD residues during the MD simulations, we observed a consistently higher number of contacts in M373I as compared to the WT model, especially considering those residues in close proximity or belonging to the SF (Figure S3). Among others, contacts with SF residues V363 and G369 were negligible in WT but noticeable in M373I (Figure 3B), suggesting a somewhat higher interaction of isoleucine with the SF region with respect to methionine, as depicted in Figure 3C, and D, a result due to the increased hydrophobicity of the former residue. In turn, residue 373 was less exposed to the solvent in the mutant than in the WT form, as shown by the large reduction of solvent accessible surface area (WT: $303 \pm 49 \text{ \AA}^2$; M373I: $171 \pm 43 \text{ \AA}^2$; Figure S4) and average number of water molecules in contact with residue 373 (WT: 11 ± 1 ; M373I: 9 ± 1). In order to investigate the effect of the mutation on the SF stability, we evaluated the average intersubunit distance between C_α atoms of residue 373 for WT and M373I models, from which we observed shorter distances in M373I than in WT, indicating a slightly tighter packing of the SF at the level of residue 373 (Figure S5). However, the most remarkable effect of M373I single mutation was observed in the change of water hydration within the SF region. From the analysis of the local water density in the SF, it was apparent the relevant dehydration effect caused by M373I mutation in this sensible portion of the channel (Figure 3E-F). Remarkably, this effect was significant not only when considering the average hydration level of the SF throughout the whole MD simulation, but also when we specifically considered ion solvation during K^+ translocation events, as displayed by the decrease in water coordination number along the channel axis whenever a potassium ion approached the SF region (Figure 3G). The loss of coordinating water molecules appeared even more significant towards the exit of the SF (about 2 water molecules), where K^+ ions become again fully hydrated and are released into the extracellular environment, thus indicating a less favorable ion translocation pathway in M373I. In the case of S390N, our functional studies revealed that the open probability of this mutant is very low, but once it is opened, it also has a lower conductance compared to the WT. However, in our MD simulation, this channel variant turned rapidly into a closed state, thus preventing any ionic current. As a consequence, no ion conductance was estimated in this case. Nevertheless, further analyses of the S390N model provided valuable insights into the origin of Kv4.3 channelopathy caused by this single mutation (*vide infra*).

Figure 3 (A) Average pore radius along the channel axial position (z-coordinate) for human WT (red) and M373I (blue) Kv4.3 TMDs embedded in a POPC lipid bilayer and simulated with an applied voltage of 1 V. A bottom view of the structural alignment of PDs for WT (red) and M373I (blue) is reported in the inset. (B) The average number of contacts of residue 373 of human WT and M373I Kv4.3 TMD with the PD region defined by residues 350-372. (C) and (D) Side views of WT and M373I SFs show the different interaction of M373 and I373 with Y360 and SF residues. SF residues for WT and M373I are highlighted in red and blue, respectively. Residues 360-366 are shown in white. M373 and I373 are shown in sky-blue and orange CPK representations, respectively. Residues in contact with M373 and I373 are shown in licorice representations. The cutoff for considering a residue in contact with residue 373 was 3.0 Å. The analysis was performed over the whole trajectory. (E) and (F) Water density volumetric maps of human WT (red) and M373I (blue) Kv4.3 SFs. Residue 373 is shown with a CPK representation in both models. The water density of the WT SF, which is represented showing only one hydration layer, is much larger than that of M373I. The volumetric maps were measured over the whole trajectory. (G) Water coordination number of K⁺ ions of human WT and M373I Kv4.3 TMDs. The SF of WT Kv4.3 is shown in silver with highlighted carbonyl groups and T367 side chains in licorice representation. Water molecules and permeating K⁺ ions (green) are shown in space filling representation. The analysis was performed over the whole trajectory.

Figure 4 The M373I and S390N mutations affect channel activation differentially in the absence and presence of KChIP2b. Steady-state activation of channels in the absence (A) and the presence (C) of KChIP2b in CHO cells. The activation currents were recorded in the whole-cell configuration and evoked by 50 ms step depolarizations from a holding potential of -90 mV to test potentials in 10 mV increments. Each pulse was followed by a fixed -50 mV step for 500 ms (see inset). Data were fit with Boltzmann equation (see Methods). WT: $V_{1/2} = -22.7 \pm 5.1$ mV, $k = 12.4 \pm 1.0$ mV ($n = 4$). M373I: $V_{1/2} = 11.1 \pm 4.8$ mV, $k = 12.0 \pm 2.0$ mV ($n = 3$). S390N: $V_{1/2} = 3.8 \pm 2.3$ mV, $k = 15.4 \pm 1.4$ mV ($n = 3$). WT + KChIP2b: $V_{1/2} = 22.7 \pm 4.2$ mV, $k = 13.0 \pm 2.8$ mV ($n = 6$). M373I + KChIP2b: $V_{1/2} = 20.3 \pm 4.8$ mV, $k = 13.3 \pm 1.5$ mV ($n = 10$). S390N + KChIP2b: $V_{1/2} = 34.8 \pm 7.1$ mV, $k = 11.1 \pm 3.7$ mV ($n = 8$). Values are expressed as mean $\pm$ SD. Macroscopic activation kinetics of WT and mutants in the absence (B) and presence (D) of KChIP2b. The time constants were obtained from fitting a single exponential term to the rising phase of the currents generated for cell conductance analysis (see Figure 2A).

2.4 M373I and S390N affect the activation differently in the presence and absence of KChIP2b

Next, we systematically studied the functional effect of the mutations at different stages of the channel gating, starting with the voltage-dependence and kinetics of the steady-state activation. The macroscopic Kv4.3 current was recorded using a holding potential of -90 mV, followed by a series of 50 ms depolarizing test potentials from -90 to +60 mV in 10 mV steps to activate the channel while minimizing inactivation (Figure 4A, inset). The mean activation curves were approximated with single Boltzmann relationships. In the absence of KChIP2b, M373I needed a higher degree of depolarization for its activation, indicating a bias towards the closed state (Figure 4A). The mean midpoint potential for activation, $V_{1/2}$, of M373I was shifted slightly to more depolarized voltages (Figure 4A and Table 1). While the $V_{1/2}$ of WT was -22.7 ± 5.1 mV ($n = 4$), that of

M373I was -11.1 ± 4.8 mV ($n = 3$). The steepness of the activation curves (slope factor, k_{act} , on the other hand, was unchanged Figure 4A), indicating that the voltage dependence of channel opening is not affected. Furthermore, the rate of activation τ_{act} , as quantified by analyzing the rising phase of peak currents generated for cell conductance analysis, was also affected. M373I-induced currents activated more slowly below -20 mV, relative to the WT (Figure 4B). When both channels were co-expressed with KChIP2b, $V_{1/2}$ of activation of M373I was indistinguishable from that of the WT (Figure 4C). KChIP2b caused a 9 mV hyperpolarizing shift in its $V_{1/2}$, compensating for the difference from the WT Kv4.3 ($V_{1/2} = -20.3 \pm 4.8$ mV, $n = 10$). For both WT and M373I, KChIP2b did not affect the rate of activation and the k_{act} (Figure 4C, D, and Table 1).

In the case of S390N alone, the channel required much larger positive voltage steps to activate than the WT and had a $V_{1/2}$ of -3.8 ± 2.3 mV ($n = 3$), indicating that this mutation strongly biases the gating towards closed states (Figure 4A). Furthermore, the rate of activation was also slow with a τ_{act} of 31.5 ± 14.4 ms ($n = 3$) compared to WT τ_{act} of 10.3 ± 1.5 ms ($n = 5$) at -30 mV (Figure 4B and Table 1). As in M373I, the k_{act} of S390N was not significantly affected; it increased from 12.4 ± 1.0 mV ($n = 4$) for the WT to 15.4 ± 1.4 mV ($n = 3$) for S390N. When S390N was co-expressed with KChIP2b, on the other hand, there were two significant effects of the mutation. First, the activation kinetics were dramatically affected such that S390N was activated as fast as the WT at all voltages (Figure 4D). Second, the $V_{1/2}$ of activation is shifted 30 mV to more hyperpolarizing voltages ($V_{1/2} = -34.8 \pm 7.1$ mV, $n = 8$) with no change in the slope factor (Figure 4C). A similar hyperpolarizing effect of an auxiliary partner on Kv4 channel half-activation voltage has been reported before. In that case, KChIP2b shifted $V_{1/2}$ of Kv4.2, which is 94% similar and 73% identical to Kv4.3, more than 20 mV to more hyperpolarized potentials, with no significant effect on the activation kinetics or slope factor, but this effect was Kv channel type dependent (An et al., 2000). Together, our results on the hyperpolarizing shift in $V_{1/2}$ of activation by KChIP2b observed only in the mutants and the unexpected effect of KChIP2b on the activation kinetics of S390N suggest that, especially in the case of S390N, the structural signatures of the channel required for its modification by KChIP2b are significantly altered.

2.5 M373I and S390N inactivate faster than WT and are differently modulated by KChIP2b

The voltage-dependence and kinetics on inactivation of Kv4 channels have a significant impact on their physiological function (Beck, Bowlby, An, Rhodes, & Covarrubias, 2002). Therefore, we next studied the effect of the mutations on the inactivation gating of Kv4.3 and its modulation by KChIP2b. To elucidate the voltage dependence of inactivation of the channels, they were activated first by 1 s pre-pulse potentials from -90 to +60 mV in 10 mV steps. During this conditioning period, the macroscopic current increased very fast and decreased exponentially. The test pulse at +60 mV directly followed the pre-pulse for another 1 s (Figure 5A, inset). The mean inactivation curves were obtained by normalizing the peak current generated by the test pulse and described by single Boltzmann relationships. Compared to the WT Kv4.3, when the channels expressed alone, no significant changes in the mean $V_{1/2}$ and the rate of inactivation were detected for M373I and S390N (Figure 5A and Table 1). Furthermore, like WT, there was also no overlap in the steady state activation and inactivation relationship (Figure S6).

Figure 5 M373I and S390N effect on Kv4.3 channel inactivation. Steady state inactivation of the WT, M373I and S390N channels in the absence (A) and the presence (B) of KChIP2b. Inactivation currents were recorded in the whole cell configuration in CHO cells from a holding potential of -90 mV to depolarized potentials in 10 mV increments, followed by a fixed +60 mV step (see inset). Data were fit with Boltzmann equation (see Methods and Table 1). (C) Exemplary whole cell currents for the WT, M373I, and S390N channels in the absence (left) and presence (middle) of KChIP2b, as recorded for cell conductance analysis (see Fig. 1). Superimposed normalized currents (evoked by depolarization to +40

mV) are shown to illustrate the effect of KChIP2b on the kinetics of each mutant (right). The scale bars show 0.5 nA (left), 1 nA (middle) currents and 100 ms. Tukey box plot summarizing the time constants (D) and their contribution (E) to the current decay at +40 mV in the absence (unfilled boxes) and presence (filled boxes) of KChIP. Horizontal lines of the boxes represent 1st, 2nd (median), and the 3rd quartile points. X presents the mean value; whiskers represent the maximum and minimum points. Symbols indicate individual data points. All symbols, lines, and boxes are color-coded: WT, red; M373I, blue; and S390N, green.

Upon co-expression of the channels with KChIP2b, the WT Kv4.3 showed 16 mV depolarizing shift in $V_{1/2}$ of inactivation (-49.6 ± 3.8 mV, $n = 5$), similar to 8-29 mV shift as reported before (Bähring et al., 2001; Patel, Parai, Parai, & Campbell, 2004) (Figure 5B and Table 1). M373I also showed a depolarizing shift of similar magnitude in its $V_{1/2}$ (Table 1). However, $V_{1/2}$ of S390N could not be modified by KChIP2b and stayed at -63.6 ± 7.8 mV ($n = 12$), resulting in channel inactivation at more hyperpolarized potentials than the WT.

To test the kinetics of channel inactivation, the macroscopic currents of WT and mutant Kv4.3 channels were generated by 2 s step depolarizations from -90 mV with 10 mV increments. In the absence of KChIP2b, the current decay for M373I was faster than for WT (Figure 5C, left panel). For a more quantitative explanation, the kinetics at +40 mV were described by an exponential function with three time constants (τ_1 - τ_3) and their fractional amplitudes (A_1 - A_3), representing the fast, intermediate, and slow phases of the current decay, respectively (Groen & Bähring, 2017). While the fast phase of inactivation of M373I was similar to that of WT, the slow and intermediate phases were faster, with significantly decreased τ_3 and τ_2 values, respectively (Figure 5D, unfilled blue and red boxes). For both channels, the corresponding relative amplitudes (A_3 - A_2) were similar and contributed significantly to the fitted decay (20 - 70%) (Figure 5E, unfilled blue and red boxes). Furthermore, the modulation of the inactivation kinetics by KChIP2b was also affected in M373I. KChIP2b differentially influences the fast and the slow phases of the macroscopic current decay of Kv4 channels (Bähring et al., 2001; Decher, Barth, Gonzalez, Steinmeyer, & Sanguinetti, 2004; Groen & Bähring, 2017). In our system, the KChIP2b-remodelled WT current decayed faster than the unmodified WT current during the slow and intermediate phases of inactivation, with significantly decreased τ_3 and τ_2 values, respectively. The decay was slower during the fast phase of inactivation with a slightly increased τ_1 (Figure 5D, filled and unfilled red boxes). The contribution of the fractional amplitude A_3 to the KChIP2b-remodeled current increased and that of A_2 and A_1 decreased as compared to the unmodified WT current (Figure 5E, filled and unfilled red

boxes). As a result, the superimposed and normalized unmodified and KChIP2b-remodelled WT currents crossed over (Figure 5C, right panel). In the case of M373I, however, all three phases of the current decay accelerated in the presence of KChIP2b (Figure 5D, filled and unfilled blue boxes). The contribution of the relative amplitude A_3 significantly increased and dominated the fractional amplitudes at this voltage (Figure 5E, filled green boxes). The normalized and superimposed M373I currents also showed the crossover, but at a later time point (Figure 5C, right panel). However, as compared to the WT, in the presence of KChIP2b, M373I inactivated slower at the slow phase and faster at the fast phase of inactivation, with relatively higher τ_3 and lower τ_1 values, respectively. Together, KChIP2b remodeling seemingly compensates the faster inactivation caused by this mutation.

The inactivation kinetics were most affected in S390N; both in the absence and presence of KChIP2b modulation, S390N channels inactivated faster (Figure 5C). In the absence of KChIP2b, the macroscopic current decayed significantly faster than the WT at all phases, with a dominant relative amplitude A_3 for the slowest time constant τ_3 (Figure 5D and E, unfilled green boxes, respectively). The development of S390N inactivation remodeled by KChIP2b exhibited a further accelerated slow phase (Figure 5D, filled green boxes) with the corresponding relative amplitude (A_3) that dominated the rest of the amplitudes (Figure 5E, filled green boxes). While τ_3 decreased significantly, τ_2 and τ_1 increased (Figure 5D) and normalized and superimposed currents had a slight crossover (Figure 5C, right panel). The corresponding relative amplitudes A_2 and A_1 decreased and did not contribute significantly to the fitted decay (<10%) (Figure 5E).

Together, our data on Kv4.3 inactivation show that M373I and S390N mutations alone do not affect the voltage dependence of inactivation of Kv4.3 but significantly accelerate the inactivation process. However, upon interacting with KChIP2b, inactivation kinetics of M373I becomes very similar to that of WT, while S390N inactivates still faster than the WT. Furthermore, while the voltage-dependence of M373I is modified by KChIP2b as expected, that of S390N is not modified, suggesting significantly altered interactions of S390N with KChIP2b.

Table 1 Gating parameters for wild-type Kv4.3 and its mutants M373I and S390N in the absence and presence of KChIP2b

	WT	M373I	S390N	WT + KChIP2b	M373I + KChIP2b	S390N + KChIP2b
Cell conductance (nS)	14.6 ± 9.7 (n = 7)	9.4 ± 9.3 (n = 14)	1.2 ± 0.9* (n = 8)	33.2 ± 15.0 (n = 7)	28.9 ± 10.9 (n = 13)	15.9 ± 8.8* (n = 16)
Single-channel conductance (pS)	11.1 ± 2.7 (n = 5)	9.5 ± 2.4 (n = 4)	9.5 ± 1.2 (n = 5)	9.6 ± 2.3 (n = 6)	9.3 ± 0.5 (n = 4)	n/a
Activation						
steady-state	$V_{1/2}$ (mV)	-11.1 ± 4.8* (n = 3)	-3.8 ± 2.3** (n = 3)	-22.7 ± 4.2 (n = 6)	-20.3 ± 4.8 (n = 10)	-34.8 ± 7.1** (n = 8)
kinetics	k_{act} (mV)	12.4 ± 1.0 (n = 4)	15.4 ± 1.4* (n = 3)	13.0 ± 2.8 (n = 6)	13.3 ± 1.5 (n = 10)	11.1 ± 3.7 (n = 8)
	τ_{act} (ms) at -30 mV	10.3 ± 1.5 (n = 5)	31.5 ± 14.4 (n = 3)	8.1 ± 0.6 (n = 4)	25.6 ± 8.5 (n = 3)	8.1 ± 1.8 (n = 4)
Inactivation						
steady-state	$V_{1/2}$ (mV)	-59.7 ± 4.0 (n = 4)	-58.1 ± 9.5 (n = 4)	-49.6 ± 3.8 (n = 5)	-43.3 ± 6.1* (n = 8)	-63.6 ± 7.8** (n = 12)
kinetics at +40 mV	k_{inact} (mV)	8.0 ± 1.4 (n = 4)	8.3 ± 3.8 (n = 4)	5.1 ± 2.4 (n = 5)	5.1 ± 1.7 (n = 8)	5.5 ± 1.4 (n = 12)
	τ_3 (ms)	168.5 ± 17.7	155.4 ± 4.4	125.4 ± 22.6	138.3 ± 29.6	101.8 ± 17.6
	A_3 (%)	61.9 ± 27.6	46.2 ± 9.6	76.4 ± 13.2	77.7 ± 4.3	84.2 ± 17.3
	τ_2 (ms)	65.0 ± 10.6	48.1 ± 7.9	49.0 ± 12.8	45.3 ± 2.1	60.7 ± 18.7
	A_2 (%)	31.6 ± 24.8	37.8 ± 5.9	20.8 ± 11.9	21.4 ± 4.0	11.3 ± 16.8
	τ_1 (ms)	9.3 ± 6.7	11.6 ± 8.2	11.2 ± 10.7	0.4 ± 0.0	8.7 ± 9.2
	A_1 (%)	6.5 ± 4.5 (n = 4)	15.9 ± 11.5 (n = 4)	2.8 ± 2.2 (n = 6)	0.9 ± 0.7 (n = 5)	4.5 ± 3.2 (n = 7)
Recovery from inactivation	τ_{rec} (ms)	397.4 ± 65.9 (n = 3)	155.6 ± 113.8 (n = 3)	478.5 ± 189.7 (n = 3)	124.1 ± 11.3 (n = 6)	153.4 ± 8.7* (n = 8)

, **, * Significantly different from the wild-type sample: $p < 0.05$, $p < 0.005$, $p < 0.0005$, respectively. $V_{1/2}$ and k stand for the midpoint voltage and slope factor. τ and A represent the decay rate and the contribution of each tau to the overall decay.*

2.6 M373I and S390N affect the recovery from inactivation

KChIPs are known to accelerate the recovery of Kv4 channels from inactivation (An et al., 2000; Bähring et al., 2001; Beck et al., 2002; Patel et al., 2004). We studied the effect of M373I and S390N on the time course of recovery from inactivation at -90 mV using a double pulse protocol (Figure 6A, inset). The channels were activated and completely inactivated with the first pulse at +60 mV for 1s. Before the second pulse at +60 mV was applied, the membrane potential was held at -90 mV for a variable interpulse interval (from 15 up to 300 ms). The second pulse reported the current recovered during the interpulse interval, namely the population of the channel that exit the closed inactive state (Figure 6A). The peak currents generated by the second pulse were normalized to the control peak current obtained by the first pulse ($I/I_{\max}$, plotted against the interpulse interval (Figure 6B), and fitted to a single-exponential function to obtain the recovery time constant (τ_{rec}).

In the absence of KChIP2b, the first pulse at -90 mV inactivated > 80% of the current at 60 mV for WT and two mutants (Figure 6B). The time constants of recovery from inactivation for WT, M373I, and S390N were not significantly different (Table 1). When the channels were co-expressed with KChIP2b, as shown before, KChIP2b accelerated the recovery of WT from inactivation with a τ_{rec} of 47.0 ± 0.9 ms, $n = 5$. However, the magnitude of acceleration was significantly lower for M373I and S390N mutants with τ_{rec} of 124.1 ± 11.3 ms, $n = 6$ and 153.4 ± 8.7 ms, $n = 8$, respectively.

In summary, our experimental data on the channel gating show that both the mild (M373I) and severe (S390N) mutations affected not only the intrinsic channel properties but also the Kv4.3 modulation by its main physiological partner KChIP2b. While the effects of M373I on Kv4.3 were milder and corrected upon interacting with KChIP2b, the effects of S390N were more severe. Even though KChIP2b interacted with S390N, it could not modulate its activity as it did that of the WT; S390N preferred to be in the closed state, inactivated faster, and recovered from inactivation slower. According to the current gating model of Kv4.3, the S6 helix, at which S390N mutation resides, plays a crucial role in channel inactivation. It has dynamic intra- and inter-subunit interactions with the S4-S5 linker

during channel gating. Wollberg et al. have identified the interaction partners important for the closed-state inactivation for a homologous potassium channel Kv4.2, which is 73.3% and 72.4% identical to Kv4.3 in its S4-S5 linker and S6 pore domain, respectively (Wollberg & Bähring, 2016). Therefore, we next investigated the origin of inactivation using our molecular model of the Kv4.3 channel.

Figure 6 The M373I and S390N mutations recover from inactivation slower than the WT. Representative traces of inactivated and recovered currents at +60 mV are shown for the WT, M373I, and S390N channels in the absence (A) and presence (C) of KChIP2b after 150 ms interpulse interval. Recovery at -90 mV holding potential developed during an interpulse interval ranging from 15 ms up to 300 ms between the two pulses (see inset), and the kinetics developed during a 1 sec pulse to +60 mV. The normalized recovered currents are shown in the absence (B) and the presence (D) of KChIP2b. The mean values of recovered currents were fitted with a single exponential (solid line). Time constants obtained at -90 mV for WT, M373I, and S390N in the absence and presence of KChIP2b are given in Table 1.

2.7 The molecular origin of S390N inactivation

The differential modulation by KChIP2b and the preference for a closed-inactive state, taken together, suggested some relevant structural changes caused by the S390N single mutation in the Kv4.3 channel. In analogy to M373I, we carried out a comparative modeling study of S390N and WT forms in an attempt to better understand the origin of the altered behavior of this mutant. Surprisingly, in our MD simulation, the S390N model turned into a closed form within the first few tens of nanoseconds and remained in this configuration since then (Figure 7A, inset). The pore

radius profile along the main protein axis displayed a markedly narrow point at the level of the gating region of the Kv4.3 channel and visual inspection of the protein structure confirmed a blocked ion pathway at the intracellular side of the channel (Figure 7A). As a consequence, no K⁺ ions were allowed to enter into the hydrophilic cavity and the SF region of the channel throughout the whole S390N simulation, in stark contrast to the WT model. Evidence of such a drastic structural change was also observed in the residue-residue contact maps of S390N as compared to WT, especially considering the increased number of contacts occurring between two opposite S6 helices, belonging to chain C and D in our model (Figure 7B and Figure S7). Accordingly, a pronounced asymmetric pairing of S6 helices contributed mostly to the observed closure of the S390N channel (Figure 7C). In order to better assess the structural modifications occurring in the S390N model, we analyzed how and to what extent the S6 helices moved within the PD. First, we noticed that in S390N the S6 helices were generally displaced towards the channel central axis, but not uniformly since the residues belonging to the helix endings, *i.e.*, residues 390-402, showed the largest deviations with respect to WT (Figure S8). As a result, the S6 helices formed inter-subunit contacts at the level of the intracellular gate region, which involves primarily residues belonging to the highly conserved PVP motif (*i.e.*, residues 398-400): in our S390N model, we noticed specifically intersubunit contacts between residues V399, P400, V401, I402, and V403. Moreover, we observed a significant increase in the S5-S6 distance within the same monomer in two of the four protein subunits (Figure S9-10), which otherwise remained well aligned in the WT form, with the largest displacements occurring around residue 390. Then, we performed the analysis of the number of contacts between residue 390 and the remaining residues of the PD, from which we noticed significant contacts with residues A331 and F335 (Figure 7D), both located on an adjacent S5 helix, and residue M365 in the H5 loop of the PD (Figure 7E). In going from WT to S390N, it was particularly apparent the increase in the number of contacts with residue A331, which is located on the S5 helix and is more closely facing residue N390 on S6: such a result was ascribed to the bulkier nature of asparagine side chain with respect to serine. In order to evaluate more specifically the effect of the mutation on the contacts mentioned above, we also analyzed 100 structures taken at even time intervals from the WT simulation, after replacing residue 390 with asparagine and, then, optimizing the N390 side chain geometry in all subunits. In this case, results showed an even starker increase in the number of contacts between residue 390 and A331 with respect to both WT and S390N simulations (Figure 7E), thus

suggesting a determining role for residue 390 in pushing S6 helix inwardly and, in turn, in inducing channel gating in the mutant. According to this view, it was expected that S390N mutation could have also exerted a destabilization effect on the S6-S5 interaction, thus triggering the observed abnormal structural changes in S390N. Indeed, analysis of the interaction energy between the S6 and S5 helices provided a clear indication of a more stable arrangement in WT as compared to S390N; the notable change in the van der Waals term of the interaction energy supported a better packing of these helical domains in the WT model with respect to S390N (Figure 7F).

Figure 7 (A) Average pore radius along the channel axial position (z-coordinate) for human WT and S390N Kv4.3 TMDs embedded in a POPC lipid bilayer and simulated with an applied voltage of 1 V at 310.15 K for 1010 ns and 350 ns, respectively. (B) Contact maps of S6 helices (residues 382-402) of human WT and S390N Kv4.3 TMDs. In S390N S6

helices exhibit closer distances compared to the same gating helices in WT and M373I models. The analysis was performed over the first 100 ns of the trajectory. (C) 250 ns snapshot showing S5 and S6 helices of human WT and S390N Kv4.3TMDs. The S390N mutant exhibits a tighter packing of S6 helices compared to WT. WT and S390N are shown in red and green, respectively. (D) 350 ns snapshot showing S5 and S6 helices of human S390N Kv4.3TMD. S5 and S6 helices are shown in transparent green to highlight the interaction of N390 with A331 and F335. (E) The average fraction of contacts of residue 390 of human WT (red) and S390N (green) Kv4.3 TMDs with S5 (residues 321-341) and H5 (residues 360-380) helices. The cutoff for considering a residue in contact with residue 390 was 3.5 Å. The same result is also shown from 100 energy minimized structures of S390N (purple), which were generated from WT structures extracted every 7 ns from the first 700 ns of the 1 μs trajectory. (F) S6/S5 helices interaction energy from human WT (red) and S390N (green) Kv4.3 TMDs.

2.8 Evidence of an altered dynamic binding mechanism in S390N

While the above results supported clear evidence for the observed preference of S390N for a closed-inactive state, its altered inactivation and recovery from inactivation kinetics also suggested a possible weaker coupling between the S6 gating helices and the VSD. Recent studies on voltage-gated potassium channels proposed a simple activation/inactivation mechanism according to which channel gating is specifically controlled by the interaction between the S4-S5 linker and the S6 helix (Wollberg & Bähring, 2016). A tight interaction (high coupling) corresponds to an active channel, which is suitable to reach an open state; a loose interaction (weak coupling), on the other hand, favors the collapse of S6 helices into the typical closed form. In our WT model, we observed that each S6 helix interacts significantly with the S4-S5 linker belonging to the same chain and partially with those belonging to other monomers (Figure S11). Interestingly, the S390N model displayed an overall reduction in the number of contacts formed between the S4-S5 linker (*i.e.*, 306-323) and the S6 helix, both inter- and intra-subunits, accompanied by a parallel increase in the intersubunit S6-S6 contacts, as a consequence of channel gating (Table S2). Again, the most striking deviations from the WT model appeared in chains C and D of the mutant, indicating the particular contribution of these two monomers to channel closing in our S390N model, as also highlighted by the contact map difference in Figure S12. In particular, only the mutant displayed S6/S6 contacts involving residues from opposite (non-adjacent) chains (*i.e.*, C and D). The residues interacting more in these two chains of the mutant were residue 399 and 400, which belong to the inner gate region of S6 (*i.e.*, PVP segment). The computed interaction energy between the S4-S5 linkers and S6 helices clearly showed a destabilization of three out of four monomers (namely chain B, C, and D) in S390N as compared to WT (Figure S13). Overall, the present results supported a weaker coupling of the S6/S4-S5 linker

binding in S390N, thus suggesting that our mutant model may represent a closed-inactive state of the Kv4.3 channel.

3 Discussion

Channelopathies are disorders caused by malfunctioning of ion channels. With recent advancements in gene sequencing technologies, many ion channel mutations have been identified and associated with human disorders. This provides the opportunity to study the disease mechanisms at the molecular level and learn also about the native physiological function in much higher detail by investigating directly the protein channels at exercise conditions. Here, we elucidate the functional and structural consequences of two neuropathogenic mutations of human Kv4.3 ion channel by employing molecular biology and electrophysiology methodologies in combination with molecular modeling and full atomistic molecular dynamics simulations.

The main effect of the physiologically mild mutation at position 373 (*i.e.*, methionine to isoleucine) is that the Kv4.3 conducts slightly less current. Our homology model and full atomistic MD simulations show that M373I and WT Kv4.3 channels are essentially structurally similar in their pore gate (P-gate) (residues 350-375) including the SF (Figure S1). In M373I, however, the presence of a more hydrophobic amino acid residue (*i.e.*, isoleucine) at the exit of the SF leads to local dehydration and to reduction of K⁺ ion permeation rate, thus lowering the overall conductance of the channel, in good agreement with electrophysiology results (Figure 3F-G and Table S1). A similar effect of hydration/dehydration around SF on channel conductance has also been observed for other ion channels (Capener, Proks, Ashcroft, & Sansom, 2003). Furthermore, M373I mutation located in the P-gate shifted the V_{1/2} activation to more depolarized voltages and accelerated the inactivation as compared to WT (Figure 4A and Figure 5C-D). In Kv4 channels, upon depolarization, the voltage sensor domain quickly moves and reaches the activation gate (A-gate) at the cytoplasmic S6 bundle crossing, but fails in making tight contact with it before drifting to a relaxed conformation. This failure of the voltage sensing domain to open the A-gate corresponds to the preferential closed-state inactivation (CSI). Thus, M373I effects on the activation and inactivation could occur as a result of an altered CSI, in which the closed-state is more stable, the interaction of the VSD with the A-gate is even more unstable, or both.

Our electrophysiology data show that M373I did not change the preference of the channel for CSI. As shown for other Kv4 channels and

the WT Kv4.3 with preferred CSI, the macroscopic currents elicited by M373I displayed no significant overlap between the voltage-dependence of activation and inactivation curves (Figure S6) (Skerritt & Campbell, 2009). Furthermore, even though we cannot exclude any potential structural constraints caused by M373I on the A-gate, which propagate from the selectivity filter downward the S5 helix and affect its interaction with the VSD, our MD simulations show that the overall M373I mutant structure has no significant differences also at the level of the A-gate when compared to the WT structure.

Another explanation might be the effect of the mutation on the P-gate of the channel. Even though seen merely in Kv4 channels, in other Kv channels, after the VSD interacts tightly with the A-gate, upon sustained depolarization, it temporarily uncouples from the A-gate, relaxes, adopt a more stable conformation and interacts with the P-gate. In some Kv channels, this interaction stabilizes the channel in a non-conducting conformation, leading to a P/C-type inactivation (Eghbali, Olcese, Zarei, Toro, & Stefani, 2002). The latter scenario is supported by the local structural changes that we observe in the M373I model around the selectivity filter region. Similar changes have been linked to the inactivation of the P-gate in potassium channels, including Kv1.2 and Kv2.1 (Matthies et al., 2016; Pless, Galpin, Niciforovic, Kurata, & Ahern, 2013). Although the inactivation of the P-gate seems to be mediated by the dilation of the pore in other channels, our model shows clear constriction and dehydration of the pore. We put forward the hypothesis that P/C-type inactivation can be exposed by mutating M373 in Kv4 channel and the structural rearrangements which underlie the inactivation of the P-gate in Kv4 channels may be different from Shaker and other Kv channels (López-Barneo, Hoshi, Heinemann, & Aldrich, 1993; Ogielska & Aldrich, 1999).

In the case of the physiologically severe mutant S390N, the mutation-induced changes were more dramatic, as expected. Functionally, each stage of channel gating, as well as the modulation of the channel by KChIP2b were significantly altered. We have found that the access of K⁺ ions to the internal gate is prevented by the tight packing of S6 gating helices, leading to a stable closed conformation (Figure 7A). The structural differences between WT and S390N channels emerge in the first few tens of nanoseconds of the simulation of the mutated system, involving especially specific hydrophobic terminal residues of S6 gating helices and more importantly those belonging to the PVP motif (residues 398- 629) (Figure 7B-C and S7, S9, S10), driving a fast closure of the

channel. The increased hydrophobic interactions of the residue 390 with specific residues of S5 (A331 and F335) and H5 (M365) helices and the reduction in S6/S5 van der Waals interactions also play a significant role in the conformational change affecting the terminal residues of S6 helices (Figure 7D-F). Moreover, the loose packing between S6 and S4-S5 linker helices accompanied by a tighter packing of S6 helices (Figure S11, S12, S13 and Table S2) leads to a closed-state inactivation of the S390N Kv4.3 channel, which is in good agreement with the dynamic binding model recently proposed for Kv4.2 channels by Wollberg et al. (Wollberg & Bähring, 2016).

Since our atomistic model of the Kv4.3 channel has successfully reproduced some experimental key results and it proved valuable for the interpretation of the effect of the two considered disease-causing mutations, we may envisage its fruitful use for further studying and for predicting other inherited and de novo mutations. Furthermore, our molecular model could be also used to shed some light on the unknown binding mode and action mechanism followed by inhibitors and blockers of the Kv4.3 channel with the final aim of developing drugs for the treatment of SCA19/22.

Finally, our study stresses the importance of taking in due consideration the role of the auxiliary partners while evaluating the physiologically relevant effects of mutations on channel function, as a mutation may not only alter structurally and functionally the ion channel itself but may also affect its activity modulation by the auxiliary proteins, as shown here.

4 Materials and Methods

4.1 *Molecular biology*

The *KCND3S* gene (wild-type, M373I, and S390N) and the gene which encoding for rat KChIP2b in the pcDNA3.1 backbone were kind gifts from Prof. D. Verbeek (Duarri et al., 2012, 2015). pmaxGFP™ (Lonza) or pEGFP-N1 (Clontech) was used to co-express a fluorescent protein as a marker for transfected cells. Chinese Hamster Ovary (CHO) cells (Sigma Aldrich) were chosen as the expression system for the lack of any intrinsic voltage-dependent electrical activity. CHO cells were maintained and grown as previously described with some modifications (Gamper, Stockand, & Shapiro, 2005). Briefly, the growth medium was Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 medium (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin (Sigma Aldrich). Cells were seeded with a density of 40.000-60.000 cells/well on a 12 mm diameter glass coverslip (Thermo

Fisher Scientific) and they were transfected after 24 hours with two (pcDNA3.1-KCND3S and pmaxGFP/pEGFP-N1) or three plasmids (pcDNA3.1 KCND3S, pcDNA3.1 KChIP2b and pmaxGFP/pEGFP-N1) using the Genius reagent (Westburg) according to the manufacturer instructions.

4.2 *Electrophysiology*

Electrophysiological recordings were performed at room temperature (18 – 22 °C) with the conventional patch clamp technique on transiently transfected CHO cells (20 – 54 hours after transfection). The bath and the pipette were filled with the external (NaCl 140 mM, KCl 4 mM, HEPES 0.4 mM, glucose 5 mM, 25 mM sucrose, CaCl₂ 1 mM, MgCl₂ 1 mM pH 7.5) and internal (KCl 140 mM, HEPES 10 mM, EGTA 10 mM, CaCl₂ 1 mM, MgCl₂ 1 mM pH 7.4, adjusted with 2.5% HCl) solutions, respectively. Different voltage protocols were applied for whole-cell recordings as detailed in the main text. Single-channel currents were elicited in the cell-attached configuration by stepping the membrane voltage from resting potential - 90 mV to + 60 mV. The cells were patched using GC120F-10 borosilicate glass microelectrodes (Harvard Apparatus, Holliston, MA) which were pulled on a P-87 puller (Sutter Instruments, Novato, CA) with a final resistance between 5 and 15 M. The data was amplified and filtered at 5kHz using an Axopatch 200B amplifier, sampled at 10 kHz in a Digidata 1320A digitizer, and analyzed with pClamp10 software (Molecular Devices). Leak current was subtracted in every file prior to any other analysis. Student's t-test was used to calculate statistical significance.

4.3 *Molecular model*

An atomistic model of the human WT Kv4.3 channel was built up using a combination of homology modeling and X-ray crystal structures, since there is a high sequence similarity among Kv channels of all families (Figure S14). Since there is a 47 % sequence identity in the transmembrane domain (TMD: helices S1-S6) between the Kv4.3 channel and the Kv2.1 paddle-Kv1.2 chimera channel, the structural model of the Kv4.3 channel was generated using the latter high resolution X-ray crystal structure as a template (PDB ID: 2R9R) (Long, Tao, Campbell, & MacKinnon, 2007). The homology model of a single monomer of the Kv4.3 channel was obtained from the SWISS-MODEL server and Swiss-PdbViewer (Benkert, Biasini, & Schwede, 2011; Bertoni, Kiefer, Biasini, Bordoli, & Schwede, 2017; Biasini et al., 2014; Bienert et al., 2017; Guex, Peitsch, & Schwede, 2009). Then, a tetrameric form of the WT Kv4.3 channel in its open state was built up from the monomeric form of the

homology model of the Kv4.3 TMD and the X-ray crystal structure of the Kv4.3 tetramerization (T1) domain, which constitutes the cytoplasmic intracellular domain (ICD) of the channel, complexed with its auxiliary subunits (PDB ID: 2NZ0) using the VMD 1.9.3 software package (Humphrey, Dalke, & Schulten, 1996; Pioletti, Findeisen, Hura, & Minor, 2006; H. Wang et al., 2007). More details on the system set up can be found in the Supporting Information.

4.4 Molecular dynamics simulations

The structure of the wild-type Kv4.3 TMD (residues 165-411) was embedded in a slightly asymmetric POPC lipid bilayer containing 443 lipids (225 and 218 POPC molecules were in upper and lower leaflets, respectively) and solvated in an aqueous solution with a 150 mM KCl salt concentration using the CHARMM-GUI Membrane Builder tool (Jo, Kim, & Im, 2007; Jo, Kim, Iyer, & Im, 2008; Wu et al., 2014). From the WT system, we generated two Kv4.3 mutant models: M373I, in the pore domain, next to the selectivity filter; S390N, in the S6 subunit. All MD simulations were performed with the NAMD 2.12 software package (Phillips et al., 2005). After following the protocol described in the Supporting Information, NVT production runs were performed at 310.15 K applying a homogeneous external electric field (E_z) along the z-axis (L_z) perpendicular to the membrane and proportional to a voltage of 1 Volt ($E_z = -1 \text{ V/L}_z$) (Chandramouli, Di Maio, Mancini, Barone, & Brancato, 2015).

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Supporting information for how do SCA19/22 mutations affect the function and structure of the wild-type Kv4.3 channel?

1 Supplementary information

1.1 Supplementary methods

1.1.1 Molecular model of the Kv4.3 channel

An atomistic model of the human WT Kv4.3 channel was built up using a combination of homology modelling and X-ray crystal structures, since there is a high sequence similarity among Kv channels of all families (Fig. S14). Since there is a ~47 % sequence identity in the transmembrane domain (TMD: helices S1-S6) between the Kv4.3 channel and the Kv2.1 paddle-Kv1.2 chimera channel, the structural model of the Kv4.3 channel was generated using the latter high resolution X-ray crystal structure as a template (PDB ID: 2R9R) (Long, Tao, Campbell, & MacKinnon, 2007a). Sequence alignments were performed with Clustal 2 using the graphical user interface ClustalX version 2.0 (Chenna et al., 2003; Higgins, Thompson, & Gibson, 1996; Jeanmougin, Thompson, Gouy, Higgins, & Gibson, 1998; Larkin et al., 2007; Thompson, 1997). The homology model of a single monomer of the Kv4.3 channel was obtained from the SWISS-MODEL server and Swiss-PdbViewer (Benkert, Biasini, & Schwede, 2011; Bertoni, Kiefer, Biasini, Bordoli, & Schwede, 2017; Biasini et al., 2014; Bienert et al., 2017; Guex, Peitsch, & Schwede, 2009) (note that part of the extracellular domain of the Kv4.3 protein is missing). Then, a tetrameric form of the WT Kv4.3 channel in its open state was built up from the monomeric form of the homology model of the Kv4.3 TMD and the X-ray crystal structure of the Kv4.3 tetramerization (T1) domain, which constitutes the cytoplasmic intracellular domain (ICD) of the channel, complexed with its auxiliary subunits (PDB ID: 2NZ0) using the VMD 1.9.3 software package (Humphrey, Dalke, & Schulten, 1996; Pioletti, Findeisen, Hura, & Minor, 2006; Wang et al., 2007). Then, the essentially non-functional T1 domain of the protein and its KChIP1 auxiliary subunits were omitted (M. O. Jensen et al., 2010, 2012; M. Ø. Jensen, Jogini, Eastwood, & Shaw, 2013). Moreover, the model of Kv4.3 TMD was validated with the Memoir server and the homology modelling algorithm designed for membrane proteins MEDELLER by using the Kv4.3 TMD as a target sequence and the Kv2.1 paddle-Kv1.2 chimera channel TMD as a template (Ebejer, Hill, Kelm, Shi, & Deane, 2013; Hill & Deane, 2013; Kelm, Shi, & Deane, 2010). The structural alignments of Kv4.3 TMDs generated with two different methods gave C α atoms RMSDs of the order of 0.2 Å. Four crystallographic K⁺ ions were placed in the selectivity filter (SF: residues 367-372) using as a reference the position of the ions in the X-ray crystal structure reported by Long et al. in 2007 (Long, Tao, Campbell, & MacKinnon, 2007b).

1.1.2 Molecular dynamics simulations

Initially, the structure of the wild type Kv4.3 TMD (residues 165-411) was embedded in a slightly asymmetric POPC lipid bilayer containing 443 lipids (225 and 218 POPC molecules were in upper and lower leaflets, respectively) and solvated in an aqueous solution with a 150 mM KCl salt concentration using the CHARMM-GUI Membrane Builder tool (Jo, Kim, & Im, 2007; Jo, Kim, Iyer, & Im, 2008; Wu et al., 2014). From the WT system, we generated two Kv4.3 mutant models: M373I, in the pore domain, next to the selectivity filter; S390N, in the S6 subunit. All MD simulations were performed with the NAMD 2.12 software package (Phillips et al., 2005). The CHARMM36 force field was used for the protein and lipids, and the TIP3P model for water (Best et al., 2012; Jorgensen, Chandrasekhar, Madura, Impey, & Klein, 1983; Klauda et al., 2010). The smooth Particle Mesh Ewald (PME) method was used to calculate the electrostatic interactions, with a short-range interaction cut-off of 12 Å using a switching function (Darden, York, & Pedersen, 1993; Essmann et al., 1995). A multiple time-step algorithm was used to integrate the equations of motion, with a time step of 4 and 2 fs for the long-range and short-range interactions, respectively, and 1 fs for the bonding interactions (Grubmüller, Heller, Windemuth, & Schulten, 1991). All bond lengths involving hydrogen atoms were held fixed using the SHAKE algorithm (Ryckaert, Ciccotti, & Berendsen, 1977). Each system was subject to the following protocol: (i) 5000 steps of energy minimization with the position of all protein and solvent atoms, ions and POPC polar headgroups fixed with a force constant of 1 kcal/mol·Å²; (ii) short NpT simulations with 0.5 fs to 2.0 fs timesteps were performed gradually releasing the harmonic positional restraints, at a constant physiological temperature of 310.15 K using a Langevin thermostat and at 1 atm pressure using a Langevin-Nosé-Hoover piston method; (iii) a 300 ns NpT equilibration simulation was carried out while keeping restrained the position of the crystallographic ions and the distances of opposing carbon atoms of the carbonyl moieties of the selectivity filter with a force constant of 10 kcal/mol·Å², followed by a 50 ns NpT equilibration releasing all restraints except those of the selectivity filter (0.25 kcal/mol·Å²) and by further addition of KCl salt up to 500 mM; (iv) NVT production runs were performed at 310.15 K applying a homogeneous external electric field (E_z) along the z-axis (L_z) perpendicular to the membrane and proportional to a voltage of 1 Volt ($E_z = -1 \text{ V}/L_z$) (Chandramouli, Di Maio, Mancini, Barone, & Brancato, 2015; Feller, Zhang, Pastor, & Brooks, 1995; Martyna, Tobias, & Klein, 1994; Starek, Freitas, Berneche, & Tobias, 2017). The channel pore radius was measured with the HOLE program (Smart,

Neduvelil, Wang, Wallace, & Sansom, 1996). Analysis of contact maps and residue-residue distances were performed with Gromacs software tools (Abraham et al., 2015). Images, movies and trajectory analyses were performed using VMD graphical tools, analytical plugins and Tcl scripts. The number of permeation events was measured with a homemade script, dividing the simulation box in three different regions, the first one at the intracellular side of the lipid membrane, the middle one at the selectivity filter level and the last one at the extracellular side of the membrane.

1.2 Supplementary results

Figure S 1 Contact maps of a region of the transmembrane PD in close proximity of the SF (residues 350-375) of human WT and M373I Kv4.3 TMDs embedded in a POPC lipid bilayer and simulated with an applied voltage of 1 V at 310.15 K for 350 ns. Contact maps are very similar for WT and M373I models. The analysis was performed over the whole trajectory.

Figure S 2 S6 helices C_{α} atoms root mean square deviations (RMSDs) of human WT (red) and M373I (blue) Kv4.3 TMDs embedded in a POPC lipid bilayer and simulated with an applied voltage of 1 V at 310.15 K for 350 ns. The average C_{α} atoms RMSD of both WT and M373I models over the time of simulation indicate that there is a remarkable similarity in the packing of S6

gating helices in the two systems. The analysis was performed over the entire trajectory for each system using as a reference the starting equilibrated structure of the WT model and averaging over the four protein chains.

Table S 1 Number of S4-S5 linker/S6 and S6/S6 contacts^a for WT and S390N Kv4.3 estimated from MD simulations.

S6 chain	WT S4-S5	S390N S4-S5	WT S6	S390N S6
A	24 (18) ^b	29 (17)	26	31
B	23 (17)	21 (14)	28	26
C	23 (17)	9 (0)	26	33
D	23 (17)	9 (7)	28	32

^a The cutoff distance to define a contact was 3.5 Å. ^b Intrasubunits contacts are reported in parentheses.

Figure S 3 Average number of contacts of residue 373 with residues 350-375 of human WT and M373I Kv4.3 TMDs embedded in a POPC lipid bilayer and simulated with an applied voltage of 1 V at 310.15 K. Each panel shows results from channel intrasubunit contacts. In M373I, the mutated residue 373 exhibits a larger average number of contacts with residues 350-375 than in

the WT model. The cutoff used for the calculation of the number of contacts was 3.0 Å. Averages and standard deviations of the number of contacts for each chain were measured over the last 40% of each trajectory.

Figure S 4 Solvent accessible surface areas (SASAs) of (A) residues 367-373 and (B) residue 373 of human WT and M373I Kv4.3 TMDs embedded in a POPC lipid bilayer and simulated with an applied voltage of 1 V at 310.15 K for 1000 ns. In the WT model the SF including residue 373 and residue 373 show larger SASAs compared to the M373I system, indicating that the presence of the mutation affects the overall SF hydration. Average SASAs for residues 367-373 and residue 373 with their errors estimated as standard deviations over the analyzed

trajectory are reported in the right panel of the figure. The probe radius used for the calculation of SASAs was 1.4 Å. The analysis was performed over the last 40% of each trajectory.

Figure S 5
Average
distances
of
residue 373 C_{α}
atoms of human
WT and M373I
Kv4.3 TMDs
embedded in a
POPC lipid
bilayer and
simulated with an
applied voltage of
1 V at 310.15 K
for 710 ns. A top
view of the SF
shows a CPK
representation of

residue 373 and the set of distances of residue 373 C_{α} s in WT (red) and M373I (blue) models. WT and M373I C_{α} s of residues 373 are shown in orange and purple, respectively. In the M373I model average distances $d1$ and $d2$ are shorter than those in the WT model, indicating a slightly tighter packing of the SF in proximity of residue 373. The average distances were measured over the last 40% of each trajectory.

Figure S 6 Normalized conductance-voltage relationships of the steady-state activation (a) and inactivation (i) of WT, M373I and S390N Kv4.3 in the absence (open symbols) and presence (closed symbols) of KChIP2b present no significant overlap.

Figure S 7 Contact maps of S6 helices (residues 382-402) of human S390N Kv4.3 TMD embedded in a POPC lipid bilayer and simulated with an applied voltage of 1 V at 310.15 K for 350 ns. In S390N S6 helices exhibit shorter distances, as compared to the same gating helices in WT and M373I models, which remain unchanged over the first 100 ns (A) and the last 250 ns (B) of the trajectory, respectively.

Figure S 8 Average C_{α} RMSD per residue of S6 helix residues from chains (A) A, (B) B, (C) C and (D) D of human WT (red) and S390N (green) Kv4.3 TMDs embedded in a POPC lipid bilayer and simulated with an applied voltage of 1 V at 310.15 K and 1 atm. The analysis and the averages were performed over 350 ns trajectories saved every 500 ps. The errors are reported as standard deviations.

Figure S 9 Average minimum distances of C_α atoms of different S6 helix (red) domains, namely 382-386, 390-394 and 398-402 (highlighted in yellow), from S5 helix (blue) of human WT (red) and S390N (green) Kv4.3 TMDs embedded in a POPC lipid bilayer and simulated with an applied voltage of 1 V at 310.15 K for 1000 ns (WT) and 350 ns (S390N). The analysis and the average were performed over trajectories saved every 50 ps.

Figure S 10 Average C_{α} atoms distance maps of S6 helices residues 391-405 of human WT and S390N Kv4.3 TMDs embedded in a POPC lipid bilayer and simulated with an applied voltage of 1 V at 310.15 K for 710 ns (WT) and 350 ns (S390N). The units of the color bar are in Å. The analysis was performed over trajectories saved every 50 ps.

Figure S 11 (A) Top and (B) side views of three chains of human WT Kv4.3 TMD showing residues of the extended S4-S5 linker (residues 306-323) in contact with S6 residues from 393 to 410. Extended S4-S5 linker, S5 (residues 321-341) and extended S6 (residues 382-411) helical domains are shown in blue, white and green, respectively. Amino acid residues of the extended S4-S5 linker in contact with residues of the terminal

domain of the extended S6 helix (residues 393-410) are shown in licorice representation. The cutoff distance to define a contact was 3.5 Å. (C) Top and (D) side views of chains C and D of WT (red) and S390N (green) Kv4.3 TMDs showing the different interaction of S6 with the S4-S5 linker.

Table S 2 Number of permeation events and conductance of K⁺ ions for WT, M373I and S390N Kv4.3 estimated from MD simulations and electrophysiological measurements at single-channel level.

Kv4.3	Permeation events	Conductance simulations (pS) ^a	Conductance experiments (pS)
WT	45	7.1	11.1 ± 2.7 (n = %)
M373I	24	3.8	9.5 ± 2.4 (n = 4)
S390N	0	-	9.5 ± 1.2 (n = 5)

^a The conductance was calculated over the entire production run. The experimental conductance values are shown together with standard deviation.

Figure S 12 The difference contact map (DCM) between human S390N and WT Kv4.3 contact maps of extended S4-S5 linker (residues 306-323) and S6 (residues 382-411) helical domains from AA MD simulations of human WT and S390N Kv4.3 TMDs embedded in a POPC lipid bilayer and simulated with an applied voltage of 1 V at 310.15 K for 1000 ns and 350 ns, respectively. In S390N S6 gating helices exhibit both shorter (blue) and longer (red) distances with S4-S5 linker

residues compared to the WT model. The analysis was performed over the whole trajectory.

Figure S 13 S6 (residues 386-407)/S4-S5 linker (residues 306-323) helices total interaction energies from MD simulations of WT (solid) and S390N (checkerboard) Kv4.3 TMDs performed for 1000 ns and 350 ns, respectively. Chains A, B, C, D are reported in red, blue, green and purple, respectively. In the S390N mutant it is observed a clear destabilization of

three chains out of four compared to the WT model. Energy values were averaged over the whole trajectory. The reported errors are standard deviations.

Figure S 14 (A) The sequence alignment of the transmembrane PD (residues 321-402) of human WT Kv4.3 with the PD of Kv1.2 from *Rattus norvegicus* shows high sequence similarity. (B) Multiple sequence alignment of the transmembrane PD (residues 359-411) of human WT Kv4.3 with different Kv channels from *Homo sapiens*. The SF domain of Kv4.3 exhibits high sequence similarity with all human Kv channels, indicating that this region of the protein is highly conserved.

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Chapter 3

De novo mutations in the Kv4.3 channel reduce the availability of native A-type current by affecting channel localization and function in mammalian cells

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This chapter is part of a larger study that will be published in a different form than presented here.

*In the brain, voltage-gated potassium channel Kv4.3 generates a fast-transient outward potassium current, known as somatodendritic subthreshold A-type current (I_{SA}). I_{SA} modulates the firing pattern of Purkinje cells by dampening the excitatory input within the dendrites. Over the last years, *de novo* mutations in KCND3 have been reported to cause cerebellar ataxia with an early age of onset and other neurological abnormalities, such as spasticity, hypotonia, and dyspraxia. How these mutations affect the channel function and structure, ultimately leading to a clinical phenotype is not yet known. Here, by using a mammalian heterologous expression system, we report on the effect of five *de novo* mutations on Kv4.3's function, localization, and structure using electrophysiology, immunocytochemistry, and molecular dynamics simulations. We found that these *de novo* mutations can be classified in two groups. The first group includes the V294F and V399L mutant channels which generated potassium currents with altered electrical activity. Specifically, the V294F mutation shifted channel conductance towards more depolarized potentials compared to WT and the main auxiliary protein KChIP2b was unable to speed up the recovery from inactivation of the V399L mutant channel as much as for the WT. The second group includes mutant channels (i.e., L310P, T366I, and G371R) which did not generate potassium currents. The L310P mutant channel is retained in the ER, both in the absence and presence of KChIP2b. The T366I mutant channel showed reduced surface expression, even in the presence of KChIP2b, but was conducting. The G371R mutant channel localizes at the plasma membrane, but was not conducting. Overall, in a heterologous expression system, these *de novo* mutations cause a reduction of I_{SA} by differentially affecting the function or localization of the Kv4.3 channel.*

1 Introduction

Ion channels are membrane proteins that allow the passive flow of ions across the cell membrane along the electrochemical gradient. These membrane proteins respond to different stimuli, such as voltage, ligand, and pressure, and play an important role in the generation of action potential and other cellular processes, including muscle contraction, cell volume regulation, hormone secretion, growth, and apoptosis. A single protein or, more commonly, several proteins, belonging to the same family, are necessary to form a functional channel. Moreover, various types of auxiliary proteins (e.g., Kv β , KChIP, and DPLP) bind to ion channels and modulate their activity (González et al., 2012).

Malfunctioning of ion channels or proteins which directly modulate ion channel activity can lead to the development of channelopathies. Since ion channels are ubiquitously found throughout the human body, channelopathies affect different biological systems (e.g., nervous, cardiovascular, and respiratory systems), their malfunctioning cause a variety of diseases, such as epilepsy, migraine, blindness, diabetes, cardiac arrhythmias, and cancer (J.-B. Kim, 2014). At least 12 genetically distinct types of spinocerebellar ataxia are caused by mutations in genes encoding for ion channels, such as voltage-gated sodium, potassium, and calcium channels. These channelopathies manifest neuronal as well as neurodevelopmental components (Klockgether, Mariotti, & Paulson, 2019; Soong & Morrison, 2018).

Mutations in *KCND3* gene have been identified to cause spinocerebellar ataxia 19/22 (SCA19/22). The first reports showed that heritable mutations in *KCND3* cause SCA19/22 – a late onset condition (Duarri et al., 2012; Lee et al., 2012). Mutant channels generated lower potassium currents compared to the WT channel. These studies showed that the lack of potassium currents resulted from retention of the SCA19/22 mutant channels within the endoplasmic reticulum (ER), leading to lower cell surface expression, and/or functional deficiencies. Interestingly, the main auxiliary protein KChIP2b rescued the effect of mutations on the channel trafficking deficiency, but did not compensate for the effect of certain mutations on channel gating (Duarri et al., 2015).

In recent years, four independent studies have also reported *de novo* mutations within the *KCND3* coding sequence (Carrasco Marina, Quijada Fraile, Fernández Marmiesse, Gutiérrez Cruz, & Martín del Valle, 2019; Hsiao et al., 2019; Kurihara et al., 2018; Smets et al., 2015). Patients who carry *de novo* mutations presented with a more severe and early onset

condition compared to SCA19/22 cases. The phenotype included not only cerebellar ataxia, but also extracerebellar symptoms, such as intellectual disability, myoclonus, and dystonia. How these *de novo* mutations affect the channel activity at the molecular level and how these changes lead to the difference in disease phenotype is not known.

Figure 1 Part of the Kv4.3 channel 3D structural model and the location of the selected *de novo* mutations (i.e., V294F, L310P, T366I, G371R, and V399L) located in different domains. The channel model is shown as grey ribbons (only two subunits for simplicity). The voltage sensor, linker, selectivity filter and A-gate are depicted in green, blue, cyan, and orange, respectively, and the mutations are shown in red. (A) Side view of the Kv4.3

channel model showing the different domains: voltage-sensing (VSD), S4-S5 linker, selectivity filter, and pore domain. (B) Top view of the channel showing the position of the de novo mutations within the domains.

The Kv4 channel is a voltage-gated potassium channel and generates a fast-transient potassium current, known as somatodendritic subthreshold A-type current (I_{SA}) in various neurons, including Purkinje neurons in the cerebellum. I_{SA} shapes the firing pattern of neuronal cells by dampening incoming stimuli and inhibiting action potential backpropagation (Connor & Stevens, 1971; J. Kim, Wei, & Hoffman, 2005). In the cerebellum, I_{SA} regulates the firing activity of Purkinje cells, which are the only efferent neurons of the cerebellar cortex.

The Kv4 channel complex is formed by the tetramerization of different Kv4 subunits (*i.e.*, Kv4.1, Kv4.2, and Kv4.3) into a heterotetramer which associates with different auxiliary proteins (*e.g.*, KChIPs and DPLPs). Kv4 channel subunits share similar structural features which are found across all Kv channels. These structural features are the voltage-sensor (formed by the first four transmembrane helices, S1-S2-S3-S4), the channel pore (formed by the last two transmembrane helices, S5-S6), the S4-S5 linker domain, and the N- and C-terminal cytoplasmic loops (Figure 1A). Each domain is essential for Kv4 to perform its function and generate the A-type current. Upon depolarization of the cell membrane, the voltage-sensing domain (VSD) physically moves and results in structural rearrangements of the linker domain, ultimately leading to the opening of the pore domain (PD). Once the channel pore is open, the selectivity filter (SF), which is encoded by a highly conserved sequence (*i.e.*, GYGD), allows the efflux of potassium ions (Birnbaum et al., 2004).

The transmembrane domains are essential for sensing voltage changes and allowing the passage of potassium ions across the cell membrane. The cytoplasmic domains are necessary for the tetramerization of subunits from the same family (*e.g.*, Kv1, Kv2, Kv3, and Kv4) and allowing the interaction with auxiliary proteins (*i.e.*, KChIPs and DPLPs) (Jahng et al., 2002). Both KChIP and DPLP generally increase the expression of the Kv4.3 channel at the plasma membrane (PM), with the exception of KChIP4a, which results in retention of the channel within the ER (Cui, Liang, & Wang, 2008; Lin, Long, Hatch, & Hoffman, 2014; Tang et al., 2013). Besides affecting the localization of the Kv4.3 channel, most KChIPs slow down channel inactivation and also increase the rate of recovery from inactivation (Patel, Parai, Parai, & Campbell, 2004).

Table 1 List of *de novo* mutations in KCND3 and clinical information.

Mutation	Inheritance	Age ¹	Clinical features	Atrophy ²	Cases
p.V294F	<i>de novo</i>	6	Ataxia	n/a	1
p.L310P	unknown	34	Cerebellar ataxia, spasticity, tremor, facial atrophy	yes	1
p.T366I	<i>de novo</i>	5	Cerebellar ataxia	no	1
p.G371R	<i>de novo</i>	4-32	Cerebellar symptoms, hypotonia, dyspraxia	no (2), n/a (1)	3
p.V399L	<i>de novo</i>	20	Cerebellar ataxia, dystonia	no	1

n/a: not analyzed ¹: age in years at latest check-up. Age range is used to show the age range of different cases. ²: presence of cerebellar atrophy based on MRI scan

Here, we report on the functional effects of five *de novo* mutations selected from a cohort of unique cases, each located in a different part of the Kv4.3 channel. First, we heterologously expressed individual mutant Kv4.3 channels in the presence and absence of KChIP2b in Chinese hamster ovary (CHO) cells and determined the effect of the *de novo* mutations on the channel function, using patch-clamp electrophysiology methods. Next, we investigated the expression of the mutant Kv4.3 channels in HeLa cells by immunocytochemistry. Finally, we performed molecular dynamics simulations in lipid environments to examine the effect of each mutation on the structure of the WT channel. We found that these *de novo* mutations resulted in a reduction of A-type potassium current at physiologically relevant membrane potentials via different mechanisms. The V294F and V399L mutations generated currents, but with altered electrical activity. Specifically, the V294F mutation shifted the A-type current to more depolarized membrane potentials, whereas the V399L mutation stabilized the closed-inactive state of the channel. The other three *de novo* mutations did not generate currents in physiological conditions. The L310P and T366I mutant channels did not generate any A-type current due to protein retention within internal membranes. Although the G371R mutant channel localized at the PM, this mutation physically blocks the pore, resulting in a non-functional channel. These results showed that, although *de novo* mutations suppressed whole-cell

potassium currents, each had a different effect on channel function and structure.

2 Results

2.1 *The three-dimensional localization of the de novo mutations*

Using the structural information from the model created by Giuseppe Brancato and coworkers (see Chapter 2), we selected five *de novo* mutations (Table 1), which were located in different parts of the channel and investigated their effect on the channel structure and function. The selected mutations included V294F, L310P, T366I, G371R, and V399L, located in the VSD, the S4-S5 linker, the entry and exit side of the SF, and the PD, respectively (Figure 1).

2.2 *The de novo mutations either abolish or significantly reduce the WT potassium current*

We transiently expressed individual Kv4.3 mutant channels in CHO cells, which do not have endogenous voltage-gated Kv4 channels, and thus provide a clean electrical background (Gamper, Stockand, & Shapiro, 2005). We first investigated the functional effects of the mutations on channel activity by expressing the channel in the absence of auxiliary protein KChIP2b. Using conventional electrophysiology experiments, the whole-cell currents from CHO cells were elicited by commanding the cell membrane from a hyperpolarized potential (-90 mV) to different depolarized voltages up to +60 mV in 10 mV steps (Figure 2A, inset). V294F (n = 3) and V399L (n = 4) mutant channels generated fast activating and inactivating whole-cell currents that were similar to WT Kv4.3 currents, albeit lower in magnitude. The whole-cell conductance of the V399L mutant channel was comparable to that of the WT, whereas the V294F mutant channel conducted significantly less compared to the WT channel (WT: 14.6 ± 9.7 nS, n = 7; V294F: 2.6 ± 1.8 nS, n = 3; $p < 0.05$). No currents could be recorded from CHO cells expressing the L310P (n = 6) and T366I (n = 5) mutant channels (Figure 2A, D).

Next, we studied the effect of the mutations on Kv4.3 channel activity modulated by KChIP2b. To do so, we transiently expressed KChIP2b together with the WT or mutant channels in CHO cells. In the presence of KChIP2b, V294F, and V399L mutant channels generated higher whole-cell currents than in the absence of KChIP2b (Figure 2B). This effect is in agreement with previous studies, where KChIP2b enhanced the surface expression and increased whole-cell currents of the Kv4.3 channel (Duarri et al., 2012). In the case of the V294F mutant channel, KChIP2b increased the whole-cell conductance from 18% up to 47% of

the WT conductance (WT + KChIP2b: 33.2 ± 15.0 nS, $n = 7$; V294F + KChIP2b: 15.4 ± 8.2 , $n = 5$; $p < 0.05$). In this situation the whole-cell conductance of the V294F mutant channel is still lower than WT, suggesting that the mutation also affects channel function and/or KChIP2b modulation of Kv4.3. In the case of the V399L mutant channel, the presence of KChIP2b caused a two-fold increase in the whole-cell conductance and reached 91% of the conductance generated by WT (WT + KChIP2b: 33.2 ± 15.0 nS, $n = 7$; V399L + KChIP2b: 30.2 ± 14.2 nS, $n = 14$) (Figure 2D). In contrast, even when co-expressed with KChIP2b, no activity was detected for L310P, T366I, and G371R mutant channels ($n = 10$, $n = 2$, $n = 26$, respectively) (Figure 2B-D).

Figure 2 Representative whole-cell currents of the WT, V294F, L310P, T366I, G371R, and V399L mutant channels, expressed (A) alone, and together with KChIP2b at (B) 37 °C and (C) 30 °C. (D) Bar graph showing whole-cell conductance of WT and mutant channels in the absence of KChIP2b (empty bars), and in the presence of KChIP2b, expressed at 37 °C (filled bars) and 30 °C (gradient bars). Statistical significance was calculated using Student's t-test (p -value: * < 0.05 , *** < 0.005). Whole-cell currents were recorded from CHO cells using patch clamp technique in the whole-cell configuration. The membrane voltage was stepped from -90 mV up to +60 mV (2 sec) in 10 mV steps. The representative whole-cell currents were recorded at +20 mV for all mutant channels, except for the V294F mutant channel which was recorded at +60 mV, to make it comparable to the other mutant. The WT, V294F, L310P, T366I, G371R, and V399L mutant channels are shown in green, blue, magenta, yellow, cyan, and red, respectively.

To investigate whether the lack of activity of the L310P, T366I, and G371R mutant channels could be due to problems in channel trafficking to the PM or channel activity, we examined first the localization of the channel by performing immunocytochemistry experiments on HeLa cells expressing the mutant channels in the absence and presence of KChIP2b. Our results

showed that, in the absence of KChIP2b, the L310P, T366I, and G371R mutant channels were not present at the PM, whereas V294F and V399L mutant channels were detected at the PM (Figure 3A, channel shown in green). KChIP2b expression partially rescued channel localization of the T366I and G371R mutant channels (Figure 3B, Kv4.3 shown in magenta). However, even in the presence of KChIP2b, no electrophysiological activity was detected for these channels. This suggests that the T366I and G371R mutations affect not only the intracellular trafficking but also the activity of the channel. In the case of the L310P mutant channel, KChIP2b expression was unable to rescue PM expression (Figure 3B). This translocation defect very likely contributes to the absence of activity from cells expressing the L310P mutant channel; therefore, it remained undetermined if this intracellular retained mutant channel is still functional.

As an alternative refolding strategy and to enhance the expression of the channel at the PM, we cultured the cells expressing the L310P and T366I mutant channels at 30 °C in the presence of KChIP2b, as previously described by Duarri *et al.* 2015, and subsequently recorded the whole-cell currents at room temperature. To check if culturing the cells at 30 °C has any effect on channel activity in general, we also expressed WT under the same conditions as the mutant channels. Culturing the cells at 30 °C did not affect the activity of the WT channel, as similar currents were detected when culturing the cells at 37 °C (Figure 2C). The T366I mutant channel was active when culturing the cells at 30 °C, however, the magnitude of whole-cell currents was significantly lower compared to WT (WT + KChIP2b expressed at 30 °C: 35.3 ± 5.9 nS, $n = 8$; T366I + KChIP2b expressed at 30 °C: 1.8 ± 0.9 nS, $n = 6$; $p < 0.0005$), suggesting fewer mutant channels at the PM, and/or a reduced channel activity (Figure 2C-D). Although the T366I mutant channel localized at the PM in HeLa, when co-expressed with KChIP2b at 37 °C, we could not record any current.

This suggests that either the T366I mutant channel is retained in CHO cells or the number of channels is low at the PM, thus, no detectable currents can be generated. Additionally, we could not detect any activity for the L310P mutant channel ($n = 19$).

A

B

Figure 3 Representative fluorescent microscopy images of WT, V294F, L310P, T366I, G371R, and V399L mutant channels transiently expressed in HeLa cells in the absence and presence of KChIP2b. (A) When expressed alone, the WT, V294F, and V399L localize at the PM, whereas the L310P, T366I, and G371R localize within internal membranes. (B) KChIP2b increases the expression level of the WT, V294F, and V399L mutant channels at the PM, and partially rescues the retention of the T366I mutant channel. The L310P and G371R mutant channels are still retained. The Kv4.3 protein is labeled with a

green/magenta (A/B) marker; KChIP2b, ER, and the nuclei are shown in green, magenta, and blue, respectively. Scale bar 10 μ m.

2.3 *The V294F mutation shifts the voltage of activation of Kv4.3*

The V294F and V399L mutant channels were the only channels generating enough currents which allowed us to perform a complete electrophysiological characterization. We started by investigating the activation properties of the V294F and V399L mutant channels. As the V294F mutation locates in the VSD of the channel, we hypothesized that the mutation would affect the activation voltage of the channel. To test if this mutation changes the activation voltage, we measured steady-state activation currents from CHO cells expressing individual mutant channels in the presence and absence of KChIP2b (Figure 4B, inset). In cells expressing the V294F mutant channel, the $V_{1/2}$ was shifted ~ 50 mV towards more depolarized voltages compared to the WT channel (WT: -22.7 ± 5.1 mV, $n = 4$; V294F: 34.2 ± 8.6 , $n = 3$) (Figure 4A, B). This indicates that the VSD would not respond to changes in the membrane potential at subthreshold potentials, as it should in the physiological conditions. On the other hand, the V399L mutation, which is located at the internal side of the pore domain, did not have any significant effect on the activation voltage, as no difference was seen compared to WT Kv4.3.

Neither V294F nor V399L mutations affected the k_{act} showing that the mutant channels respond to membrane voltage changes at the same rate as the WT (Figure 4A). As for the WT channel, KChIP2b did also not have significant effects on the steady-state activation of the V294F and V399L mutant channels (Figure 4B).

Second, we investigated the effect of the V294F and V399L mutations on the channel activation kinetics. As shown by Patel *et al.* 2004, WT Kv4.3 activated slow ($\tau_{act} = 10.3 \pm 1.5$, $n = 5$, at -30 mV) at hyperpolarized voltages and fast at depolarized voltages ($\tau_{act} = 2.0 \pm 0.4$ ms, $n = 5$, at $+60$ mV) (Figure 4C, D). Both V294F and V399L mutant channels activated slower than WT at $+60$ mV ($p < 0.05$) in the absence of KChIP2b; but KChIP2b was able to rescue this effect (Figure 4C).

Figure 4 Steady-state and kinetics of activation for the WT, V294F, and V399L mutant channels transiently expressed in CHO cells in the presence and absence of KChIP2b. The *de novo* V294F shifts the activation voltage of the WT channel, while the V399L mutation has no effect on channel activation. Steady-state activation curves are shown in the absence (A) and presence (B) of KChIP2b. Data were fit using Boltzmann equation to estimate the voltage and rate of activation. WT: $V_{1/2} = -22.7 \pm 5.1$ mV, $k = 12.4 \pm 1.0$ (n = 4). V294F: $V_{1/2} = 34.2 \pm 8.6$ mV, $k = 9.6 \pm 4.9$ (n = 3). V399L: $V_{1/2} = -19.8 \pm 3.2$ mV, $k = 11.7 \pm 2.5$ (n = 6). WT + KChIP2b: $V_{1/2} = -22.7 \pm 4.2$ mV, $k = 13.0 \pm 2.8$ (n = 6). V294F + KChIP2b: $V_{1/2} = 33.9 \pm 5.0$ mV, $k = 8.8 \pm 1.5$ (n = 8). V399L + KChIP2b: $V_{1/2} = -16.2 \pm 8.5$ mV, $k = 14.2 \pm 3.7$ (n = 9). Values are expressed as mean $\pm$ S.D. Activation kinetics in the absence (C) and (C) the presence (D) of KChIP2b. The time constants were obtained by fitting a single exponential to the rising phase of whole-cell currents, as recorded for cell conductance (see Figure 2, inset). Steady-state activation currents were recorded in the whole-cell configuration by stepping the voltage from -90 mV (maintained for 500 ms) up to +60 mV in 10 mV step for 50 ms. Each pulse was followed by a fixed 500 ms step at -50 mV (see inset).

2.4 The V294F and V399L mutant channels inactivate differently compared to the WT

To test the effect of the V294F and V399L mutations on the inactivation of the channel, we recorded the whole-cell currents elicited using a standard steady-state inactivation protocol on CHO cells (Figure 5B, inset). When expressed without KChIP2b, the V294F mutant channel inactivated at a more depolarized membrane voltage compared to the WT (V294F = -7.5 ± 12.2 mV (n = 3); WT = -59.7 ± 4.0 mV (n = 4) (Figure 5A). This ~ 50 mV shift is in the same direction and similar in magnitude to the one observed for the steady-state activation. The V399L mutant channel did not show any significant difference in inactivation compared to the WT.

Interestingly, the presence of KChIP2b could shift the inactivation voltage towards more depolarized membrane potentials and increasing the sensitivity to changes in membrane voltage for both WT and V399L mutant channels, but not for the V294F mutant channel (Figure 5A-B).

Figure 5 Steady-state and kinetics of inactivation for the WT, V294F, and V399L mutant channels transiently expressed in CHO cells in the presence and absence of KChIP2b. The effect of the V294F and V399L de novo mutations on the inactivation of the Kv4.3 channel. Steady-state inactivation curves are shown for the WT, V294F, and V399L mutant channels in the absence (A) and the presence of KChIP2b (B). Data were fit using Boltzmann equation to estimate the voltage and rate of activation. WT: $V_{1/2} = -59.7 \pm 4.0$ mV, $k = 8.0 \pm 1.4$ (n = 4). V294F: $V_{1/2} = -7.5 \pm 12.2$ mV, $k = 12.3 \pm 7.7$ (n = 3). V399L: $V_{1/2} = -55.7 \pm 3.6$ mV, $k = 8.2 \pm 1.1$ (n = 6). WT + KChIP2b: $V_{1/2} = -49.6 \pm 3.8$ mV, $k = 5.1 \pm 2.4$ (n = 5). V294F + KChIP2b: $V_{1/2} = 10.9 \pm 11.8$ mV, $k = 5.4 \pm 2.0$ (n = 8). V399L + KChIP2b: $V_{1/2} = -46.6 \pm 6.2$ mV, $k = 5.4 \pm 1.5$ (n = 6). Values are expressed as mean $\pm$ S.D. (C) Superimposed normalized currents (evoked by depolarization to +60 mV) are shown to illustrate the effect of KChIP2b on the kinetics of each mutant. Dotted and solid lines represent the channel currents in the absence and presence of KChIP2b, respectively. Tukey box plot summarizing the time constants (D) and their contribution (E) to the current decay at +60 mV in the absence (unfilled boxes) and presence (filled boxes) of KChIP2b. Horizontal lines of the boxes represent 1st, 2nd (median), and the 3rd quartile points. Whiskers represent the maximum and minimum points. Symbols indicate individual data points. All symbols, lines, and boxes are color-coded: WT, green; V294F, blue; and V399L, red. Steady-state inactivation currents were recorded in the whole-cell configuration by stepping the voltage from -90 mV (maintained for 500 ms) up to +60 mV in 10 mV step for 1 s. Each pulse was followed by a fixed 1 s step at +60 mV (see inset).

Next, we investigated whether these *de novo* mutations have any effect on the inactivation kinetics of the channel which inactivates in three phases (*i.e.*, slow, intermediate, and fast). The contribution (A_i) of each phase and its absolute value (τ_i) determine the overall current decay. For example, a decrease in the contribution of the fast phase will result in slow-inactivating currents, and *vice versa*. In order to quantify these components, the current decay at +60 mV was described with a three-term exponential function, where τ_3 , τ_2 , and τ_1 represented the slow, intermediate, and fast time constants, and A_3 , A_2 , and A_1 their corresponding contribution to the decay. In the absence of KChIP2b, the V294F and V399L mutations did not have any significant effect on the channel inactivation kinetics (blue, red, and green solid lines in Figure 5C, respectively). In the presence of KChIP2b, the slow phase of inactivation accelerated for the WT, as previously reported, this was not observed for the V294F mutant channel, which was slow with a higher value of τ_3 compared to WT. Furthermore, for the V294F mutant channel, τ_2 contributed more to the decay compared to WT, as shown by an increase in A_2 , while τ_1 contribution (A_1) went below 10% resulting in a monophasic fast inactivation (Figure 5C-E). The V399L mutation did not have any significant effect on the inactivation kinetics of the channel, whereas KChIP2b was unable to accelerate the slow phase of inactivation (τ_3) of the V399L mutant channel as much as for the WT channel (WT + KChIP2b τ_3 : 184.2 ± 49.4 ms, $n = 4$; V399L + KChIP2b: 373.2 ± 154.3 ms, $n = 10$; $p < 0.005$) (Figure 5C-E). Overall, these mutations change the modulation of the channel by KChIP2b which cannot slow the inactivation kinetics of the channel, resulting in the loss of overall channel conductance.

2.5 *The V294F and V399L mutations alter the recovery from inactivation of the WT channel*

Given the prominent role of the Kv4.3 channel on delaying and suppressing action potentials in neurons, we investigated the effect of the V294F and V399L mutations on the recovery from inactivation of the WT channel. Currents were recorded from CHO cells expressing the WT and mutant channels individually, and stimulated the transfected cells with a voltage pulse at +60 mV from a – 90 mV holding potential (maintained for 300 ms). The depolarizing voltage step, which activated all channels in the membrane, was followed by a second pulse at +60 mV applied at varying time intervals from 50 to 300 ms. This allowed us to quantify the fraction of active channels at the PM (Figure 6A).

Table 2 The effect of the de novo V294F and V399L mutations on the cell conductance and gating of the WT Kv4.3 channel

	WT	V294F	V399L	WT + KChIP2b	V294F + KChIP2b	V399L + KChIP2b
Cell conductance (nS)						
	14.6 ± 9.7 (n = 7)	2.6 ± 1.8* (n = 3)	6.3 ± 7.9 (n = 4)	33.2 ± 15.0 (n = 7)	15.4 ± 8.2* (n = 5)	30.2 ± 14.2 (n = 14)
Act.						
V_{half} (mV)	-22.7 ± 5.1	34.2 ± 8.6***	-19.8 ± 3.2	-22.7 ± 4.2	33.9 ± 5.0*	-16.2 ± 8.5
k (mV)	12.4 ± 1.0 (n = 4)	9.6 ± 4.9 (n = 3)	11.7 ± 2.5 (n = 6)	13.0 ± 2.8 (n = 6)	8.8 ± 1.5 (n = 8)	14.2 ± 3.7 (n = 9)
T_{act} (ms) at +60 mV	2.0 ± 0.4 (n = 5)	8.5 ± 3.7* (n = 4)	3.0 ± 0.6*** (n = 9)	2.3 ± 0.2 (n = 4)	2.8 ± 1.1 (n = 10)	2.8 ± 0.7* (n = 13)
V_{half} (mV)	-59.7 ± 4.0	-7.5 ± 12.2*	-55.7 ± 3.6	-49.6 ± 3.8	10.9 ± 11.8***	-46.6 ± 6.2
Inact.						
k (mV)	8.0 ± 1.4 (n = 4)	12.3 ± 7.7 (n = 3)	8.2 ± 1.1 (n = 6)	5.1 ± 2.4 (n = 5)	5.4 ± 2.0 (n = 8)	5.4 ± 1.5 (n = 6)
$T_{inact-3}$ (ms)	507.1 ± 258.0	458.0 ± 238.5	649.6 ± 476.6	184.2 ± 49.4	1228.2 ± 985.7	373.2 ± 154.3**
$T_{inact-2}$ (ms)	121.7 ± 48.2	104.9 ± 56.0	120.7 ± 52.0	154.3 ± 95.3	71.1 ± 18.0	168.7 ± 55.5
$T_{inact-1}$ (ms)	13.7 ± 24.4	3.2 ± 4.4	31.3 ± 46.8	5.7 ± 10.0	0.5 ± 1.0	13.4 ± 23.5
$A_{inact-3}$ (%)	24.2 ± 11.7	18.3 ± 11.0	26.8 ± 14.1	52.0 ± 36.2	7.4 ± 11.4	23.0 ± 25.8
$A_{inact-2}$ (%)	66.3 ± 18.2	77.0 ± 11.9	63.8 ± 17.0	48.0 ± 36.2	92.6 ± 11.3	74.5 ± 26.9
$A_{inact-1}$ (%)	9.5 ± 19.0 (n = 4)	4.7 ± 3.1 (n = 4)	9.4 ± 13.9 (n = 12)	0.0 ± 0.0 (n = 4)	0.0 ± 0.0 (n = 10)	2.5 ± 7.0 (n = 10)
Rec. from inact.						
T_{rec} (ms)	397.4 ± 65.9 (n = 3)	109.8 ± 11.4 (n = 4)	8.7 10 ⁹ ± 1.8 10 ⁸ *	47.0 ± 0.9 (n = 5)	23.1 ± 21.2 (n = 8)	131.5 ± 34.8* (n = 5)

*, **, *** Significantly different from the wild-type sample: $p < 0.05$, $p < 0.005$, $p < 0.0005$, respectively. $V_{1/2}$ and k stand for the midpoint voltage and slope factor. τ and A represent the decay rate and the contribution of each tau to the overall decay.

We measured the amount of recovered current at 90 ms after the first pulse. We found that the V294F mutant channel generated more current than the WT channel: 55% and 17% of the initial current, respectively (Figure 6A). Surprisingly, this difference did not have any significant effect on the rate of recovery from inactivation, possibly due to the lack of data points at time intervals from 0 to 90 ms (Figure 6C). Although the V399L mutant channel generated similar currents compared to the WT channel, the V399L mutation significantly slowed down the rate of recovery from inactivation (WT: 397.4 ± 65.9 ms, $n = 3$; V399L: 8689650688 ± 181080256 ms, $n = 8$, $p < 0.05$) (Table 2). Overall, the presence of KChIP2b speeded up the recovery from inactivation for all three channels; WT, V294F, and V399L. We found that the V294F mutant channel is slightly, but not significantly, faster than the WT. For the V399L mutant channel, KChIP2b was unable to speed up the recovery of inactivation as much as for the WT (WT + KChIP2b: 47.0 ± 0.9 ms, $n = 5$; V294F + KChIP2b: 19.3 ± 1.1 , $n = 8$, not significant; V399L + KChIP2b: 123.8 ± 14.3 ms, $n = 5$, $p < 0.05$) (Figure 6D). Thus, the V399L mutant channel is overall less active compared to the WT channel.

Figure 6 Recovery from inactivation kinetics for the WT, V294F, and V399L mutant channels transiently expressed in the presence and absence of KChIP2b. The V294F and V399L mutant channels recover from inactivation faster and slower compared to WT, both in the presence and absence of KChIP2b. Representative traces of inactivated and recovered currents at +60 mV are shown for the WT, V294F, and V399L channels in the absence (A) and presence (B) of KChIP2b after 100 ms interpulse interval. Recovery at -90 mV holding potential developed during an interpulse interval ranging from 15 ms up to 300 ms between the two pulses (see inset), and the kinetics developed during a 1 sec pulse to +60 mV. The normalized recovered currents are shown in the absence (C) and

the presence (D) of KChIP2b. The mean values of recovered currents were fitted with a single exponential (solid line). Time constants obtained at -90 mV for WT, V294F, and V399L mutant channels in the absence and presence of KChIP2b are given in Table 2.

2.6 *The T366I mutation causes a widening of the selectivity filter, while the G371R mutation blocks the passage of ions across the pore*

For the T366I and G371R mutant channels on which we could not employ electrophysiological characterization, we employed molecular dynamics simulations to study the effect of the mutations on channel conductance and structure. We did not include the L310P mutant channel in the MD analysis, as up to now it remained unclear whether the L310P mutant channel forms a tetramer.

Briefly, the Kv4.3 channel model (described in Chapter 2) was placed into a lipid bilayer, and single-channel conductance, pore radius, and various structural changes (e.g., amino acid contacts and structural changes) were monitored over time upon cell membrane depolarization. We found that the T366I mutant channel had a higher single-channel conductance (22.7 pS) compared to the WT channel (7.1 pS). This high single-channel conductance could be explained by a widening of the pore radius at the level of the SF (Figure 7A). Moreover, the S6 helix was slightly more mobile in the T366I mutant channel compared to the WT (Figure 7B).

In the case of the G371R mutation, the mutant channel was functional in the MD analysis but exhibited a single-channel conductance of 1.7 pS compared to 7.1 pS of the WT channel. To investigate if this reduction in conductance could be due to a change in the pore size radius, we generated a pore radius profile and a contact map between the different amino acids in and around the channel pore. The G371R mutant channel pore size was reduced in the radius of the A-gate, and more contacts were observed between the S6 helices (Figure 7A, C). Additionally, the SF was more packed in the G371R mutant channel compared to the WT; likely the result of an increased number of interactions between arginine residues in this region (Figure 7C). The decreased radius and the introduction of a charged side chain at the exit of the pore due to the presence of an arginine at position 371 may explain the loss of conductance of the G371R mutant channel.

3 Discussion

Together, our functional investigations showed that *de novo* mutations in the Kv4.3 channel cause a loss-of-function. This is reflected by a marked reduction of the A-type potassium current resulting by different alterations

in the function and/or localization of the Kv4.3 channel. Based on our results, we can classify *de novo* mutations in two groups based on the ability of the mutant channel to generate currents in physiological conditions (*i.e.*, 37 °C). The first group includes the V294F and V399L mutations that correspond to mutant channels which are able to generate potassium currents, although exhibit altered functional properties. The second group is constituted by L310P, T366I, and G371R mutations, that are linked to channels that are not able to generate any currents in physiological conditions.

Figure 7 Pore radius profile and contacts maps for the WT, T366I, and G371R mutant channels. (A) The T366I mutant channel has a wider pore radius at the level of the selectivity filter compared to the WT. The G371R mutation causes a narrowing of the pore radius at the level of the A-gate and the selectivity filter. The pore radius is shown in green, cyan, and magenta for the WT, L310P, and G371R mutant channels, respectively. (B, C) Contact maps show that the T366I and G371R mutant channels are overall structurally similar. The S6 helix is more mobile in the T366I mutant channel compared to WT, and the G371R mutant shows more contacts between arginine residues at position 371 (compare interactions between AC, AD, and BC chains).

Of the first group, the V294F mutation that is located in the VSD affected the voltage sensitivity of the Kv4.3 channel. Previous studies have shown

that mutations within the VSD affect the channel responsiveness to changes in membrane voltage. For instance, in early studies, the neutralization of charged and non-charged amino acids (located within the S4 transmembrane helix) has been proven to shift the activation voltage of Shaker channel (Aggarwal & MacKinnon, 1996; Carvalho-de-Souza & Bezanilla, 2018; Seoh, Sigg, Papazian, & Bezanilla, 1996). A similar effect has been observed for Kv4.3, where the activation voltage was shifted due to a *de novo* duplication of the RVF triplet located within the S4 helix (Smets et al., 2015). Moreover, mutations within the VSD have been shown to affect both activation voltage and recovery from inactivation in Kv4.3 (Skerritt & Campbell, 2007, 2009). Our results support the idea that the VSD plays a role, not only in determining voltage sensitivity, but also in modulating the recovery from inactivation in Kv4.3 channels. Moreover, it corroborates the idea that the neutralization of, not only charged amino acids but also non-charged ones (*i.e.*, V294F) affects the functioning of the Kv4.3 channel.

We found that the V294F mutation causes a shift in the activation and inactivation of the channel towards more depolarized membrane potentials and leads to the development of a clinical phenotype with an early onset (Table 1). This is similar to functional effect (shift in the generation of potassium current of about ~60 mV) and the age of onset caused by a previously reported *de novo* mutation (*i.e.*, dupRVF293-295) (Smets et al., 2015). Together these observations suggest that the RVF motif may be a hotspot for mutations leading to the development of a clinical phenotype. Moreover, mutations in the VSD in general, specifically in the S4 domain, may share a common mechanism of action. Since all mutations located in the VSD do not entirely abolish the A-type potassium current, instead shift the current beyond subthreshold membrane potentials.

The second mutation in the first group, the V399L mutation, is located in the PVPV motif, and caused the channel to recover more slowly from inactivation compared to WT. The PVPV sequence is part of the PD domain of Kv4 channels and serves as a filter which allows the passage of potassium ions. Furthermore, the PVPV motif constitutes the channel internal gate (*i.e.*, A-gate), which governs the opening and closing of the channel by stabilizing the contact between the S4-S5 linker with the S6 helix. In Kv4.1, individual (*i.e.*, PVPI) and tandem (*i.e.*, PIPI) mutations within the PVPV domain incrementally slow the inactivation of the channel, while the same mutations speed up the recovery from inactivation (Jerng, Shahidullah, & Covarrubias, 1999). In the case of

Kv4.3, both the kinetics of inactivation and recovery from inactivation were slowed down by mutating the PVPV motif into PIPV even the presence of KChIP2b, supporting the role of the A-gate in channel inactivation (Wang et al., 2002). In our study, the V399L mutation had similar effects as the PIPV mutation. When expressed together with KChIP2b, the V399L mutant channel recovered more slowly from inactivation compared to WT.

In the second group of *de novo* mutations, we showed that the L310P mutation completely suppressed channel conductance, very likely the result of channel retention within the endoplasmic reticulum. This data suggests that this mutation has a significant role in the folding and stability of the Kv4.3 channel. Interestingly, some of the heritable SCA19/22 mutations (*i.e.*, Δ F227, T352P, M373I, and S390N) also caused protein instability and misfolding that could be rescued by culturing the cells at 30 °C (Duarri et al., 2015). However, we were unable to rescue the L310P mutant channel by culturing the cells at low temperature. We may speculate that the substitution of L310 to proline may hinder the folding of the Kv4.3 channel subunit by breaking the α -helical domain in which L310 resides. Substitutions of amino acids to proline have already been shown to affect the structure and protein folding ability, as was reported for Mip-like peptidyl-prolyl *cis-trans* isomerase (rFKBP22) (Polley, Chakravarty, Chakrabarti, Chattopadhyaya, & Sau, 2015). Moreover, given that we were not able to use MD simulations or perform electrophysiology recordings on the L310P mutant channel we advocate for the need to develop a method to investigate the electrical activity of the Kv4.3 channel in a cell-free environment (see Chapter 6).

In the second group, we were able to use MD simulations to investigate the functionality of the T366I and G371R mutant channels. Our MD results showed that both mutations affected the pore size radius and interactions among S6 helices. The G371R mutation blocks the passage of ions across the channel pore due to steric hindrance leading to absence of potassium currents. On the contrary, the T366I mutation causes an increase in single-channel conductance, suggesting that the T366I mutant fails to generate potassium currents due to protein retention within the ER and/or altered electrical activity. The G371R mutation was reported in this study for three different cases (Table 1). In another study, the G371R mutation has also been found in a 13-month old child affected by motor dysfunctions (Carrasco Marina et al., 2019). These independent observations and our functional data strengthen the pathogenicity of this specific genetic variant.

We have found that different mutations located within the same protein domain affect similar aspects of channel functioning. First, the V294F mutation, located in the VSD, primarily affects the voltage sensitivity of the channel. Qualitatively, the same effect has been reported for another genetic variant (dupRVF293-295) sharing the same physical location with V294F (Smets et al., 2015). Second, mutations located around the SF, such as T366I and G371R, affect single-channel conductance. Moreover, we have reported that the M373I mutation, also located within the SF, affects single-channel conductance (Chapter 2). Third, the V399L mutation, located at the A-gate, affects recovery from inactivation. Similarly, the S390N mutation has been shown to affect recovery from inactivation (Chapter 2). This confirms the role of the VSD, SF, and A-gate in modulating voltage sensitivity, single-channel conductance, and channel inactivation.

Overall, we have shown that *de novo* mutations affect the function, localization, and structure of the WT Kv4.3 channel in a different manner but in the end, all result in an overall reduction of the A-type potassium current at subthreshold membrane potentials. The fact that both *de novo* and heritable mutations in *KCND3* result in an overall reduction in potassium currents, cannot explain the difference in clinical phenotypes between the *de novo* cases and the SCA19/22 patients (Kurihara et al., 2018; Smets et al., 2015; Takayama et al., 2019). This difference may be unraveled by studying the effect of these mutations on neuronal firing and in the presence of other auxiliary proteins that are specifically expressed in cerebellar neurons (e.g., Purkinje cells).

4 Acknowledgments

We would like to thank all patients, clinicians, and clinical geneticists that participated.

5 Materials and methods

5.1 Patient cohort description

Patients were identified over a 4-year period through routine genetic diagnostics service in the diagnostic centers in Utrecht, Nijmegen and Groningen in the Netherlands. To collect more patients with *de novo* mutations in *KCND3*, a request was submitted to GeneMatcher (<https://genematcher.org>) (Sobreira, Schiettecatte, Valle, & Hamosh, 2015). The study design is in line with the diagnostic request and thus no informed consent is required.

5.2 *Molecular biology*

The pcDNA3.1-KCND3 coding for the short isoform (NM_172198) of the WT Kv4.3 channel was used as a template for the introduction of the *de novo* mutations (V294F, L310P, T366I, G371R and V399L) using site-directed mutagenesis (see primers list) (Duarri et al., 2012). The pcDNA3.1-KChIP2b plasmid coding for the rat KChIP2b auxiliary protein was obtained as previously reported. The plasmid sequence was checked by Sanger sequencing.

5.3 *Cell culture transfection*

Chinese Hamster Ovary (CHO) cells were grown as previously reported with some modifications (Gamper et al., 2005). Briefly, cells were grown in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 medium (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin (Sigma Aldrich). For transfection, 40.000-60.000 cells/well were seeded on a 12-mm glass coverslip, allowed to grow for ~24 hours and then transfected using the Genius reagent (Westburg) according to the manufacturer instructions. Cells were simultaneously transfected with pmaxGFP/pEGFP-N1 from Lonza/Clontech (used to select for transfected cells) along with pcDNA3.1-KCND3S alone or together with pcDNA3.1-KChIP2b.

5.4 *Immunocytochemistry*

Immunocytochemistry was performed as described previously (Duarri et al., 2012). Images were obtained by a DMI 6000 inverted microscope (Leica Microsystems).

5.5 *Electrophysiological recordings*

Electrophysiological recordings were performed as described in Chapter 2, see Materials and Methods section. Different voltage protocols were applied for whole-cell recordings as detailed in the main text and figure legends.

5.6 *Molecular dynamics simulations*

The molecular model of the Kv4.3 channels and the molecular dynamics simulations were performed as described in Chapter 2, see Materials and Methods section.

5.7 *Data analysis*

The cell conductance analysis was performed by extrapolating peak current values at different membrane voltages (from - 90 to + 60 mV). The peak current values (from - 30 to + 60 mV and, only for the V294F mutant both in the presence and absence of KChIP2b, from + 10 to + 60

mV) were fitted with linear regression and the slope value was taken as the cell conductance. The steady-state activation and inactivation curves were obtained by taking the maximum sweep current value at the third pulse for every membrane voltage. This value was normalized to the maximum current value recorded within the third pulse. The $V_{1/2}$ and the slope factor were obtained by fitting the activation and inactivation curves with a single Boltzmann equation. The activation and inactivation kinetics were calculated by fitting the sum of one or three exponential to the activation and inactivation currents. For the activation kinetics, the exponential fit was performed over the rising phase of the current (from 35% up to 100%). For the inactivation kinetics, the exponential fit was performed on the decaying phase of the current from the point with the steepest decline to the end of the sweep. The recovery from inactivation analysis was performed by extrapolating the peak current values in the fourth pulse. These were divided by the peak current value in the second pulse from the same sweep. Leak current was subtracted in every single file prior any other analysis. Student's t-test was used to calculate statistical significance using MS Excel (Microsoft). Box plots were produced using R. Datapoints from raw data was extrapolated using ClampFit10 (Molecular Devices).

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Chapter 4

Using the unnatural amino acid ANAP to label the Kv4.3 channel: unlocking the potential to perform structure-function study

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Different structural conformations determine the functioning of ion channels allowing for the binding of ligands or the movement of ions across the cell membrane. Snapshots of static structural conformations (e.g., open or close) have been visualized using, e.g., X-ray crystallography, cryo-EM, and NMR, however, these techniques do not always provide information regarding the dynamic changes from one structure to another. In order to gain information about the real-time structural dynamics of ion channels, voltage-clamp fluorometry (VCF) can be used. This technique, in combination with fluorescent probes, allows following local structural rearrangements during channel activity at virtually any position on the protein. Here, we tested the feasibility of following dynamic structural changes of Kv4.3 around its voltage sensor by genetically introducing a fluorescent unnatural amino acid (i.e., 3-(6-acetylnaphthalen-2-ylamino)-2-aminopropanoic acid) to seven positions in its voltage-sensing domain and employed VCF for studying the structural rearrangements during channel gating in response to membrane potential. We found that ANAP can be introduced at some of the targeted positions, but not all. The introduction of ANAP was less tolerated at residues located deep into the transmembrane domain and less bulky than ANAP. Moreover, the fluorescence changes could not be detected due to the low number of fluorophores at the plasma membrane. This study shows that it is feasible to use ANAP labeling for studying the structural dynamics of the Kv4.3 channel, while it highlights some of the limitations which need to be taken into account when designing future studies.

1 Introduction

Membrane proteins play a crucial role in the communication between the outside and inside compartments of cells. Specifically, ion channels are ubiquitous membrane proteins found in all living organisms (*i.e.*, prokaryotes, archaea, and eukaryotes) and their main function is to allow the passage of ions across an otherwise impermeable cell membrane. As any other membrane proteins, ion channels perform their function by adopting different structural conformations, such as active, inactive, open or close conformation. The movement from one conformation to the next allows ion channels to alternate the opening of a binding pocket towards external signaling molecules (*e.g.*, membrane lipid, blocker, inhibitor), to open a pathway for the conduction of ions by sensing voltage or pressure changes, or to become insensitive to certain external stimuli by entering an inactive conformation.

In the case of Kv4 channels (*i.e.*, Kv4.1, Kv4.2, Kv4.3), a class of voltage-gated potassium channels, four subunits from the same family come together to form a functioning channel. Kv4 channels are sensitive to membrane voltage changes in the subthreshold range (Isbrandt et al., 2000). Furthermore, their function can also be modulated by several external stimuli, such as membrane lipids, small ligands, and other regulatory proteins (*e.g.*, KChIPs and DPLPs) (Bougis & Bougis, 2015; Jerng & Pfaffinger, 2014; Tang et al., 2013; Wang et al., 2007). Once the Kv4 channel complex opens, potassium ions flow along the electrochemical gradient from the inside to the outside of the cell. This flow generates a fast, transient outward potassium current, known as the A-type potassium current, which is essential for the proper functioning of neurons.

From the alignment of the amino acid sequence between the Kv4 channels and other voltage-gated potassium channels (Kvs), it is clear how the Kv4 channel family is structurally very similar to other Kvs. In chapter 2, we show the main structural elements of Kv4.3 on an atomistic model of the protein, as our international collaborators developed at the beginning of this project. Each subunit is made up of cytoplasmic N- and C-termini and six transmembrane domains. The first four transmembrane domains form the voltage-sensing domain (VSD) that is responsible for detecting voltage changes across the cell membrane, while the last two transmembrane domains form the channel pore (PD) that opens up a path inside the channel for K⁺ ions to flow through (González et al., 2012).

Although the general structural architecture of the Kv4 channel is similar to other voltage-gated ion channels, its structural dynamics are quite different from that of Kv1 and Shaker channels, which are known to prefer an open-inactive conformation over a closed-inactive one (Bähring & Covarrubias, 2011). When the membrane is at rest, the Kv4 channel resides in its closed resting state (Figure 1, resting state). At this stage, the voltage-sensing domain (VSD) is responsive to changes in the membrane potential; when the membrane depolarizes, the channel moves into a closed-active state, which is permissive for opening but not yet conducting (Figure 1, closed-active state). From here, the channel can go into two states; an open conducting state, if the VSD can form a stable contact with the pore domain (PD) via the S4-S5 linker, and a closed-inactive state, if the contact between the linker and the PD is not strong enough (Figure 1, open and closed-inactive states). Eventually, the Kv4.3 channel goes back to its closed resting state (Bähring & Covarrubias, 2011; Fineberg, Szanto, Panyi, & Covarrubias, 2016).

How the structural dynamics of membrane proteins is intertwined with their functional role, as in the case of the Kv4 channel, is an exciting key question for mechanistic understanding of ion channels' function. That is why, over the years, scientists have developed and applied different techniques to gain information regarding the structural conformations of membrane proteins. Among these techniques are X-ray crystallography, electron microscopy (EM), and nuclear magnetic resonance (NMR). X-ray crystallography offers the possibility to determine the three-dimensional localization of amino acids for ion channels in different fixed states (e.g., ligand-bound, open, inactive) and it has been used to unravel the first atomic structure of a bacterial potassium channel (KscA) and a mammalian voltage-gated potassium channel (Kv1.2) in an open conformation (Doyle et al., 1998; Long, Campbell, & Mackinnon, 2005). Although a growing number of crystal structures have been solved, the formation of well-organized crystals limits the application of this technique, especially to membrane proteins. An alternative technique, which does not require the formation of crystals, has come to the forefront in the past few years: cryo-electron microscopy (cryo-EM). This technique has been used to solve the structure of different ion channels, such as the transient receptor potential (TRP) channel, whose structure had never been crystallized in its whole length, and other channels, such as the calcium-activated chloride channel TMEM16A, the Orai channel and the glutamate AMPA receptor (Cao, Liao, Cheng, & Julius, 2013; Liao, Cao, Julius, & Cheng, 2013; Liu et al., 2019; Paulino, Kalienkova, Lam, Neldner, & Dutzler, 2017; Zhao, Chen, Swensen, Qian, & Gouaux, 2019). However,

the drawback of cryo-EM is that it cannot be used on low molecular weight complexes (*i.e.*, approximately 100 kDa).

Another available technique is nuclear magnetic resonance (NMR), which can be applied to study proteins up to 100 kDa and, as well as cryo-EM, does not rely on the formation of crystals. Using NMR, the binding site of natural and synthetic blockers (*i.e.*, kaliotoxin and porphyrin) has been characterized for the chimeric potassium channel KscA-Kv1.3, different conformational landscapes have been identified for the selectivity filter of the bacterial potassium channel KcsA, and the calmodulin-mediated gating of the Kv7.2 channel has been investigated (Ader et al., 2009, 2008; Bernardo-Seisdedos et al., 2018; Jekhmane et al., 2019).

These three techniques have been very helpful to shed more light on identifying different conformational states that membrane proteins are in. However, they do not necessarily provide information on the structural dynamics of the channels. In order to overcome this limitation, it would be ideal to follow the structural dynamics of membrane proteins real-time, for instance by using site-specific engineered probes, namely spin labels and fluorophores, whose high spatio-temporal resolution signal could be followed using electron paramagnetic resonance (EPR) and fluorescence spectroscopy (FS), respectively.

Figure 1 A schematic depiction of the Kv4 channel structural dynamics. The Kv4 channel is found in its resting state (R) after the cell membrane has hyperpolarized. When the cell membrane depolarizes, the voltage-sensing domain (VSD) harnesses the electrical energy to pull the S4-S5 linker outwards (closed-active). If all four linkers can form a stable contact with the pore domain (PD), the channel will open; otherwise the channel will end up in its closed-inactive (CI) state. EC and IC label the extracellular and intracellular side of the cell membrane.

Fluorescence spectroscopy has been used in combination with two-electrode voltage clamp (TEVC), known as voltage clamp fluorometry, to simultaneously observe structural and functional changes which take place at the amino acid level in ion channels (Talwar & Lynch, 2015). This technique has been used to study the movement of the VSD in Shaker channel by labeling amino acids that are located on the extracellular side using different probes, such as tetramethylrhodamine-maleimide, fluorescein-5-maleimide, and Oregon Green-5-maleimide (Cha & Bezanilla, 1997; Mannuzzu, Moronne, & Isacoff, 1996). The same labeling strategy has also been used in combination with the patch-clamp technique to gain insight on how structural rearrangements of the cytoplasmic domain can transfer to a transmembrane domain in the cyclic nucleotide-gated channel (Kusch & Zifarelli, 2014; Taraska & Zagotta, 2007; Zheng & Zagotta, 2000). As can be expected from the labeling strategy of introducing the probe from outside the cell, these applications have three major limitations: (i) the structural changes can only be observed for the extracellular domains of the channels; (ii) the amino acid residue and the fluorescent probe need to chemically react with each other; and iii) as the probes are usually linked to the protein by using the free thiol groups of cysteine residues, the probe applied externally also binds to any other proteins with exposed free cysteine groups, generating nonspecific signals.

These limitations can be overcome by using genetically encoded fluorescent amino acids (Figure 2) (Chatterjee, Guo, Lee, & Schultz, 2013; Lee, Guo, Lemke, Dimla, & Schultz, 2009). This strategy allows labeling at any desired position on the protein backbone and eliminates the need for protection of already existing free cysteine residues or for introduction of engineered cysteines to allow probe binding.

Figure 2 The voltage clamp fluorometry technique. (A) A depiction of the labeling process. The different molecular components which are necessary to label the Kv4.3 channel with the fluorescent unnatural amino acid (ANAP) are depicted: genetically engineered

aminoacyl-tRNA synthetase (aaRS), tRNA (Chatterjee et al., 2013), and 3-(6-acetylnaphthalen-2-ylamino)-2-aminopropanoic acid (ANAP). The mRNA contains an amber stop codon (TAG) which encodes for ANAP. The cell membrane is depicted in grey, and the ribosome is colored in red. (B) The schematic representation of the set-up which is used to simultaneously record the fluorescence changes of ANAP and the electrical activity of the Kv4.3 channel.

The unnatural amino acid ANAP (*i.e.*, 3-(6-acetylnaphthalen-2-ylamino)-2-aminopropanoic acid) has been used to label the Shaker channel and voltage-sensing phosphatase from *Ciona intestinalis* (CiVSP), and structural changes could be followed while measuring the protein activity (Kalstrup & Blunck, 2013; Sakata, Jinno, Kawanabe, & Okamura, 2016). In the first study, the authors labeled two intracellular sites, which were not accessible by click chemistry labeling, and the fluorescent signal could be used to infer the gating mechanism of the Shaker channel linking the movement of specific structural domains with channel activity. The second study has used an unnatural amino acid to study the motion of the CiVSP catalytic region, and has concluded that the catalytic region moves in a voltage-dependent manner and into different conformational states depending on the degree of activation.

Figure 3 Chemical formulas of ANAP and the substituted natural amino acids. The chemical structures of ANAP (top), valine (bottom left), serine (bottom center), and alanine (bottom right) are shown. The side chain of ANAP is visibly bulkier than the one of the substituted amino acids.

In this chapter, we evaluated the feasibility of employing unnatural amino acids for monitoring the conformational changes of the Kv4.3 channel while it moves from a closed to an open conformation. To this end, we

tested one of the most important and mobile parts of the channel, *i.e.*, the voltage sensor S4 helix. We expressed the channels in oocytes and showed that the channel can be genetically labeled using ANAP (Figure 3), and that the efficiency of the labeling depends on the targeted position.

2 Results

2.1 Selection of residues for the incorporation of ANAP in Kv4.3

Seven residues (*i.e.*, Val282, Ser283, Gly284, Ala285, Phe286, Val287, Thr288), which are located at the N-terminus of the S4 transmembrane domain in the Kv4.3 channel, were selected for the incorporation of ANAP within the protein backbone (Figure 4). We chose amino acids within the S4 transmembrane domain because, when the Kv4 channel activates, this is the region that undergoes large structural rearrangements. As can be seen in Figure 4, Val282 and Ser283 are positioned on a loop structure at the top of the S4 transmembrane domain and face the extracellular environment, whereas the other five residues form the N-terminal region of the S4 helix. Among these residues, Gly284, Ala285, Val287, and Thr288 are in contact with the lipid environment, while Phe286 is surrounded by other transmembrane helices belonging to the same subunit.

Figure 4 Selected residues for the incorporation of ANAP in Kv4.3 channel. (Top left) Top-view of the Kv4.3 channel structural model (see Chapter 2), which is formed by four identical α subunits. (Top right) Zoomed-in side-view of one subunit around the selected amino acids. (Bottom) Sequence alignment of Shaker S4 and human Kv4.3 channel. S3 and S4 transmembrane domains are highlighted in yellow, and the selected residues are highlighted in green. Ala359 (in Shaker channel) and Ala285 (in Kv4.3 channel) are highlighted in red.

2.2 ANAP can be genetically incorporated in the Kv4.3 channel

We labeled the individual residues located in the VSD by injecting oocytes with mRNA bearing an *amber* stop codon (*i.e.*, tag) at the selected position, together with DNA carrying the necessary information to generate ANAP-loaded tRNA and ANAP. Next, we assessed channel activity of the full length ANAP-labeled Kv4.3 channel by measuring the ionic currents using two-electrode voltage clamp (TEVC).

By performing TEVC, we showed that ANAP could be individually incorporated at Val282, Ser283, and Ala285 without affecting channel functioning. We also recorded some currents slightly above the baseline from the Phe286 and Thr288 ANAP-labeled Kv4.3 channels. However, we did not consider these samples further in our analysis due to the low number ($n < 2$) of replicates (Figure 5A).

Channels labelled with ANAP at Val282, Ser283, and Ala285 generated WT-like transient fast-activating and fast-inactivating outward currents. The activation voltage of these ANAP-labeled Kv4.3 channels was about -30 mV, as was observed for wild-type Kv4.3 (B). However, the peak currents decreased from 16.7 ± 4.6 μ A ($n = 3$) for the wild-type channel to 4.2 ± 2.2 ($n = 5$), 1.7 ± 1.2 ($n = 2$) and 0.6 ± 0.3 μ A ($n = 2$) for Val282, Ser283 and Ala285, respectively (Table 1). This suggests that either the surface expression of these ANAP-labeled channels is significantly lower than wild-type, and/or ANAP at the given positions significantly alters channel activity. The activity of Phe286- and Thr288-ANAP was minimal and Gly284- and Val287-ANAP Kv4.3 channels did not produce any current (Figure 5A). Both Gly284 and Val287 face the same side of the helix.

While we could incorporate ANAP at a number of positions (*i.e.*, Val282, Ser283 and Ala285) on Kv4.3; we could not record any changes in ANAP fluorescence (Figure 5C). In order to rule out any technical issues, we performed the same procedure on the voltage-sensitive phosphatase CiVSP, which was labeled with ANAP, and used as positive control. We could record the expected change in fluorescence signal of ANAP for CiVSP using the same experimental setup and the same batch of ANAP label that we used for Kv4.3 channel. This data suggests two possible scenarios. In one case, ANAP would be functional, but its fluorescence would not change during channel activation. In another case, the number of fluorophores would be too low to detect any change in ANAP fluorescence.

Using the unnatural amino acid ANAP to label the Kv4.3 channel

Figure 5 ANAP labeled Kv4.3 channel. (A) Val282/Ser283/Ala285/Phe286/Thr288ANAP-labeled Kv4.3 channel activity. One representative trace for each ANAP-labeled position is shown. (B) ANAP-labeled Kv4.3 channel activity resembles wild-type one. Normalized peak current of wild-type (blue), Val282ANAP (orange), Ser283ANAP (red), and Ala285ANAP (yellow) Kv4.3 are plotted. The voltage activation threshold for Val282ANAP, Ser283ANAP and Ala285ANAP Kv4.3 channels is about -30 mV, similar to the wild-type channel. (C) No changes in ANAP fluorescence are detected for

Val282ANAP/Ser283ANAP/Ala285ANAP-labeled Kv4.3 channel. Data are collected in parallel with electrophysiological recordings. Gaussian filter is applied with a 300 Hz cut-off. Fast transient outward currents are elicited by voltage clamp protocol: the holding potential is held at -100 mV, and the membrane voltage is depolarized from -80 mV to $+80$ mV with a 10 mV increment. The cell membrane is depolarized for 500 ms (see voltage profile at the bottom-right corner of panel A).

2.3 ANAP incorporation efficiency depends on the side chain size and amino acid location

We showed that ANAP can be genetically incorporated within the backbone of Kv4.3 at positions Val282, Ser283, and Ala285, without affecting the channel sensitivity to voltage (Figure 5B). These ANAP-labeled channels generated lower currents compared to WT channel (Figure 6A). Since the intensities of these currents varied depending on the position at which ANAP was inserted, we hypothesized that ANAP substitution is differently tolerated depending on the targeted residue.

Figure 6 The surface expression of ANAP-labeled Kv4.3. (A) Elicited currents (at $+80$ mV) are shown for the Val282/Ser283/Ala285ANAP-labeled Kv4.3 samples. The currents were obtained using the same voltage protocol as in Figure 5A. (B) The surface expression negatively correlates with the depth at which ANAP is incorporated within the transmembrane domain. Val282ANAP-labeled Kv4.3 shows the relative highest expression level, whereas Ala285ANAP-labeled Kv4.3 shows the lowest expression level. Ser283ANAP-labeled Kv4.3 shows an intermediate expression level. These three residues line up along the vertical axis of the S4 helix from top to bottom: Val282 (top), Ser283 (intermediate) and Ala285 (bottom) (as shown in Figure 4). (C) The surface expression positively correlates with the substituted side chain size. (D) The surface expression does not show a strong correlation with the side chain hydrophobicity. The hydrophobicity values are arbitrary for pH 7 (Monera, Sereda, Zhou, Kay, & Hodges, 1995). The mean peak currents recorded at $+80$ mV is plotted against the amino acid

position at which ANAP is incorporated and the substituted side chain size. Data from two independent experiments are shown for Val282ANAP (Experiment 1 $n = 4$ and Experiment 2 $n = 5$), while data from one experiment are shown for Val283 ($n = 2$) and Ala285 ($n = 2$).

As shown in Figure 4, Val282 and Ser283 are located within a flexible loop, and Ala285 is situated within an α -helical domain. To investigate if there was a correlation between the amino acid position and the surface density of the ANAP-labeled Kv4.3 channels, we looked at the surface expression of the channels (*i.e.*, the maximum cell current) as a function of the labeled residue's depth on the z-axis of the membrane, the hydrophobicity of the amino acid, and the bulkiness of the substituted amino acid relative to ANAP (Figure 6B-D). We found a negative correlation between the plasma membrane expression level of ANAP-labeled Kv4.3 and the depth of the ANAP-labeled residue within the plasma membrane, where ANAP is incorporated (Figure 6B). The channels with ANAP incorporated at the most external (Val283) and internal position (Ala285) generate the highest ($4.2 \pm 2.2 \mu\text{A}$) and lowest ($0.6 \pm 0.3 \mu\text{A}$) ionic current, hence surface density of the ion channel, respectively, while the intermediate position has an intermediate value between the two ($1.7 \pm 1.2 \mu\text{A}$).

We also found a positive correlation between the surface expression and the difference in chain bulkiness of the original amino acid compared to ANAP. The substituted residues whose side chain is much smaller than ANAP yield the lowest current (Figure 6C), suggesting that bulkier substitutions such as ANAP in these positions disturb the protein structure. We did not find a strong correlation between the hydrophobicity of the side chain of the substituted amino acid and the surface expression of ANAP-labeled channels (Figure 6D). Overall, our data show that ANAP substitution in Kv4.3 is less tolerated at positions that are located deeper in the S4 helix, and when the substituted amino acid was less bulky than ANAP.

3 Discussion

In this study we tested the feasibility of *in vivo* labeling of Kv4.3 with an unnatural amino acid for structure-function studies. We could successfully incorporate ANAP into different positions of the transmembrane voltage-sensing domain and showed that the insertion of ANAP is more tolerated at positions that are close to the extracellular surface than positions within the transmembrane domain of the protein. This phenomenon has also been observed for other proteins. For instance, in T4 lysozyme (T4L), the incorporation efficiency of spin-labeled amino acid is lower within the transmembrane domain than within extracellular domains (Cornish et al.,

1994). A screening of suitable positions for the incorporation of two unnatural amino acids (*i.e.*, p-azido-L-phenylalanine, azF and p-benzoyl-L-phenylalanine, BzF) in the human serotonin transporter (hSERT) showed that the incorporation of azF and BzF is functionally less tolerated within the transmembrane helices than in the loops or the termini. Only 44% and 20% of the hSERT mutants, where azF/BzF is introduced within the transmembrane segments, retain functional activity, whereas the majority of the hSERT mutants (100% and 95% for azF/BzF), where azF/BzF is introduced within the terminal region, retain the function (Rannversson et al., 2016).

Table 1 The surface expression of wild-type and ANAP-labeled Kv4.3 channel.

ANAP position		Kv4.3 surface expression (μA)		
		24 h	48 h	72h
WT		n/a	16.66 ± 4.58 (n = 3)	n/a
Val282	Exp. 1	1.18 ± 0.8 (n = 3)	3.18 ± 1.57 (n = 4)	n/a
	Exp. 2	n/a	4.16 ± 2.23 (n = 5)	n/a
	Exp. 3	n/a	n/a	2.97 (n = 1)
Ser283		n/a	1.68 ± 1.16 (n = 2)	n/a
Ala285		n/a	0.64 ± 0.27 (n = 2)	n/a

Peak currents from wild-type (measured at clamped voltage = + 75 mV), Val282ANAP, Ser283ANAP and Ala285ANAP (measured at clamped voltage = + 80 mV) Kv4.3 channel.

We could not observe channel activity when we substituted Gly284 and Val287 with ANAP. The lack of activity in voltage clamp experiments could be the result of non-functional channels or no channels in the cell membrane, as a result of *e.g.*, protein retention in the endoplasmic reticulum or a deficiency in the trafficking of the channel to the cell membrane. Several SCA19/22-mutated Kv4.3 channels (*i.e.*, T352P, M373I and S390N) show trafficking deficiencies and retention in the endoplasmic reticulum (Duarri et al., 2012). The trafficking deficiency of these mutants can be rescued by lowering the temperature from 37 °C to 30 °C or by co-expressing auxiliary subunits (*e.g.*, KChIP2b) along with the Kv4.3 mutant (Duarri et al., 2015). In our experiments, the temperature

was already low; the expression of ANAP-labeled Kv4.3 took place at 18 °C. In the future, in order to increase the expression at the plasma membrane, the ANAP-labeled Kv4.3 channel may be expressed together with other auxiliary subunits (*i.e.*, KChIP2b and DPLPs).

Table 2 Comparison of the expression level and fluorophore count for different ANAP-labeled proteins.

	Maximum current/charge	Num. of channels (10 ⁶)	Num. of fluorophores (10 ⁶)	Ref.
ANAP- labeled Kv4.3	3.18 ± 1.57 µA	0.67	2.68	This study
CiVSP	16 nC*	145500*	145500*	(Murata <i>et al.</i> , 2005)
Shaker	30 nC*	23000*	92307*	(Roux <i>et al.</i> , 1998)
Wild-type Kv4.3	16.66 ± 4.58 µA	3.47	(13.88**)	This study

*: protein expression values are taken from studies where the CiVSP and Shaker channel are not labeled with ANAP.

**: this is the predicted number of fluorophores, whether the expression level of ANAP-labeled Kv4.3 could be brought to its maximum, currently achieved by the expression of wild-type Kv4.3.

The different incorporation efficiencies of ANAP at different positions may be explained by different hydrophobicity and side chain bulkiness between the substituted amino acid and ANAP. Hydrophobicity of ANAP and the substituted amino acids did not strongly correlate with the incorporation efficiency (Figure 6C), but, interestingly, the side chain bulkiness did (Figure 6D). This suggests that the side chain bulkiness has a major impact on Kv4.3 channel stability. However, ANAP could not be efficiently incorporated in place of Phe286, which exhibits similar features as ANAP; it is both hydrophobic and bulky compared to the other tested amino acids. This low incorporation efficiency may be due to the specific position that Phe286 has within the channel structure. We know, from the model, that the side chain of this residue is buried within a highly packed region between two α -helices. The tolerance to amino acid substitution

has been shown to be low, when an hydrophobic residue (in our case Phe), located far from the protein surface, is mutated to a another hydrophobic residue (in this case ANAP) (Farheen, Sen, Nair, Tan, & Madhusudhan, 2017).

After successful incorporation of ANAP into the backbone of ion channels, the gating-induced structural changes could be reported for other ion channels, *i.e.*, Shaker channel (Kalstrup & Blunck, 2013). However, we could not detect any fluorescence changes in Kv4.3. While one reason could be different structural rearrangements between the Kv4.3 and Shaker channel, a more likely explanation is the significantly low number of fluorophores, in other words the number of ANAP-labeled channels at the plasma membrane. In our studies, the number of fluorophores was thousands of times lower than that reported in other studies (Table 2) (Kalstrup & Blunck, 2013; Murata, Iwasaki, Sasaki, Inaba, & Okamura, 2005). For future studies aiming at following structural dynamics in Kv4.3 using ANAP, the channel density in the membrane should be enhanced.

Another possibility is that the absence of fluorescence is due to the expression of unlabeled Kv4.3 (*i.e.*, leak expression). This may result from the insertion of any random residue in place of ANAP or from the ribosome skipping the stop codon and continue with translation (*i.e.*, ribosomal slippage) (Herr, Nelson, Wills, Gesteland, & Atkins, 2001; von der Haar & Tuite, 2007). The efficiency of ribosomal slippage depends on complex combinations of stop codon identity, sequence context, RNA structure, and relative abundance of tRNAs. Readthrough events have been reported in *Xenopus laevis* oocyte (Bienz, Kubli, Kohli, de Henau, & Grosjean, 1980; Bienz et al., 1981). In our case, although our data show that ANAP can be incorporated within the Kv4.3 channel, we do not know whether we have a mixed population of ANAP-labeled and unlabeled Kv4.3 channel (Kalstrup & Blunck, 2015; Leisle, Valiyaveetil, Mehl, & Ahern, 2015). Whether the ionic current, which is recorded from our sample, is partly generated by Kv4.3 leak expression needs further investigation.

Overall, this study shows that the Kv4.3 channel can be genetically labeled using the fluorescent unnatural amino acid ANAP. Although we could not detect any change in ANAP fluorescence, our results prove the feasibility to use such a methodology to introduce probe for structure-function studies of the Kv4.3 channel. At the same time, some limitations of this methodology have been tested and may be useful to design future experiments.

4 Materials and methods

4.1 Plasmid construction and cRNA synthesis

The *KCND3S* gene (coding for the short isoform, 636 amino acids, of Kv4.3, NM_172198.2) was cloned into the empty pSD64TF vector (designed by Dr. Terry Snutch and provided by Dr. Yasushi Okamura) using XhoI and NotI restriction sites. The mutation from sense to *amber* stop codon (*i.e.*, 'tag') within the *KCND3S* sequence was achieved by performing round PCR on the newly created pSD64TF-*KCND3S* vector, according to the QuickChange® manual (Stratagene). The primers are listed in Table 3. Standard molecular biology protocols were used for the manipulation of the nucleic acid (Wood, 1983). pAnap, a plasmid that encodes tRNA and aminoacyl-tRNA synthetase, was kindly provided by Peter G. Schultz (The Scripps Research Institute, La Jolla, CA). The cRNA synthesis was carried out according to the "mMESSAGE mMACHINE kit" from Ambion®. The plasmids (*i.e.*, pSD64TF-*KCND3S* and mutated pSD64TF-*KCND3S*) were linearized using XbaI (Toyobo Co., Ltd., Japan).

4.2 *Xenopus laevis* oocytes and ANAP incorporation

Adult female *Xenopus laevis* was anesthetized for 15' by placing it into an ice-box, filled with ice-chilled water supplemented with 1 g ethyl 3-aminobenzoate methanesulfonate salt. Ovarian lobules were surgically removed from the abdomen of the animal through a short (about 2 cm long) vertical incision. The harvested oocytes were reduced to clumps by manual dissection. Individual oocytes were obtained by incubation in 0.1% collagenase (SigmaAldrich) dissolved in ND96(+) solution (5 mM HEPES, 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, pH 7.5, supplemented with 10 µg/ml gentamycin and 5 mM sodium pyruvate) at room temperature on a rotatory shaker for 4-5 hours. The oocytes were washed in ND96(+) and incubated for half an hour on a rotatory shaker. The harvested oocytes were stored at 18 °C in ND96(+) solution (Boulter and Boyer, 2001).

An arbitrary number of oocytes were sorted based on quality. The follicular membrane was removed by passing the oocytes several times through a narrow glass pipette tip until the membrane was loose in solution. The oocytes were placed in a 60-well plate (1 oocyte/well) with the animal pole facing upwards and were centrifuged for 10'-15' at 400-600 x g to allow nucleus exposure. 23 nl of pAnap (10 ng/µL) were injected in the nucleus. After 24 h the oocytes were injected with 50 nl cRNA-ANAP solution (either 1 ng/µl *KCND3S* cRNA or 10ng/µl CiVSP cRNA mixed with 0.5 mM 3-(6-acetylnaphthalen-2-ylamino)-2-aminopropanoic acid (ANAP),

previously dissolved in DMSO) and incubated at 18 °C in the dark. Nucleic acid injection were performed using a micro-injector (Narishige, Japan).

4.3 ANAP fluorescence measurement, electrophysiological recording and data analysis

Electrophysiological recording and fluorescence imaging were simultaneously performed. Currents were recorded with a two-electrode voltage clamp (TEVC) setup 1-3 days after cRNA injection. The pipettes were filled with 3 M KCl and had a resistance between 0.6 to 1 MΩ. ND96(+) was used as the bath solution. Currents were elicited by depolarizing the membrane potential from – 80 mV to + 80 mV with a 10 mV step increase. – 100 mV was used as holding potential. ANAP fluorescence was recorded as previously reported (Sakata *et al.*, 2016).

Table 3 Primer overview

ID	Sequence (5' → 3')	Usage
General cloning		
XhoI_KCND3_Fwd	AAAAA <i>ctcgag</i> ACCATGGCGGCCGAGATT	
NotI_KCND3-STOP_Rev	TTTTT <i>gcggccgc</i> TTACAAGGCGGAGACCTT	
Amber stop codon mutation		
ANAP_Kv43_V282_F	GAGGACtagTCCGGCGCCTTCGTC	Val282ANAP
ANAP_Kv43_V282_R	GCCGGAActaGTCCTCGTTGTTGGT	
ANAP_Kv43_S283_F	GACGTGtagGGCGCCTTCGTCACG	Ser283ANAP
ANAP_Kv43_S283_R	GGCGCCctaCACGTCCTCGTTGTT	
ANAP_Kv43_G284_F	GTGTCCtagGCCTTCGTACGCTC	Gly284ANAP
ANAP_Kv43_G284_R	GAAGGCctaGGACACGTCCTCGTT	
ANAP_Kv43_A285_F	TCCGGCtagTTCGTACAGCTCCGG	Ala285ANAP
ANAP_Kv43_A285_R	GACGAActaGCCGGACAGTCCTC	
ANAP_Kv43_F286_F	GGCGCctagGTCACGCTCCGGGTC	Phe286ANAP
ANAP_Kv43_F286_R	CGTGACctaGGCGCCGGACACGTC	
ANAP_Kv43_V287_F	GCCTTtagACGCTCCGGGTCTTC	Val287ANAP
ANAP_Kv43_V287_R	GAGCGTctaGAAGGCGCCGGACAC	
ANAP_Kv43_T288_F	TTCGTtagCTCCGGGTCTTCCGC	Thr288ANAP
ANAP_Kv43_T288_R	CCGGAGctaGACGAAGGCGCCGGA	

Current traces were analyzed using ClampFit10.7 (Molecular Devices, LLC.). The peak current at any given clamped voltage was extrapolated. Currents were normalized against the highest current (recorded at + 80 mV) within the same trace. The normalized currents were averaged and the standard sample error was calculated using LibreOfficeCalc (The Document Foundation, Berlin).

The pictures of Kv4.3 3D model were created using UCSF Chimera (the Regents, University of California) based on the model provided by Giuseppe. GIMP (The GIMP Development Team) software was used for graphic manipulation of figures.

5 References

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Chapter 5

The methionine residue at the exit of the pore affects single-channel conductance of the Kv4.3 channel

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The voltage-gated potassium channel Kv4.3 generates a fast-transient A-type current which is important for the regulation of cardiac and neuronal activity. In the open state, Kv4 channels allow the passage of potassium ions across the pore domain at a constant rate (i.e., conductance). This rate is ultimately determined by the selectivity filter which is a domain formed by a highly conserved sequence (TVGYGD), shared across potassium channels in all kingdoms of life. In Kv4.3, the SCA19/22 mutation p.M373I caused dehydration of the channel pore, thus hindering the passage of ions and leading to a decrease in single-channel conductance. We tested the hypothesis that the introduction of a hydrophilic amino acid (glutamic acid) at position 373 would result in an increase in single-channel conductance using a combination of electrophysiology and molecular dynamics simulations. Our results showed in a functional and structural manner how residues at the exit of the pore affects single-channel conductance in Kv4.3. Moreover, our data supports the usage of the Kv4.3 model to predict the effect of patient mutations reported in case studies in silico and to modify channel conductance.

1 Introduction

Ion channels are membrane proteins which are essential for the generation and propagation of electrical signals along the neuronal body. These channels can be activated by different stimuli, such as ligand, voltage, and pressure, and allow the passage of different ionic species (e.g., potassium, sodium, and calcium). Up to date, more than 30 genes encoding for voltage-gated potassium (Kv) channels have been identified and have been divided into 12 families based on structural similarities (González et al., 2012).

Functional Kv channels are formed from the assembly of four subunits from the same family. These channels have transmembrane and cytoplasmic domains. The transmembrane domains are responsible for sensing changes in the membrane potential and physically coupling the resulting conformational changes to the opening of a pore through which the potassium ions go through. The cytoplasmic domains are essential for the formation of functional tetrameric complexes and regulate the channel activity. For instance, the N-termini constitutes the binding site for one of the auxiliary proteins (*i.e.*, Kv channel-interacting proteins, KChIPs, and dipeptidyl peptidase-like proteins, DPLPs). These are proteins that modulate the channel gating as well as the trafficking of the channel to the plasma membrane (PM).

The Kv4 channel family is formed by three members: Kv4.1, Kv4.2, and Kv4.3. These members show high sequence similarities and, as in the case of other Kv channels, form heterotetrameric functional channels, and auxiliary proteins interact with the channel to shape its activity. *In vivo*, the Kv4 channel complex generates a fast-transient potassium current, known as A-type potassium current, which activates at membrane potentials that are lower than the potential required to generate an action potential, *i.e.*, subthreshold potentials. In neurons, this current delays the firing of action potential and inhibits action potential backpropagation by hyperpolarizing the membrane potential back to the resting potential (Jerng & Pfaffinger, 2014).

Potassium channels, including voltage-gated ones, are 1.000 times more likely to allow the passage of potassium ions over sodium ions. This high selectivity is encoded in the amino acid sequence (*i.e.*, TVGYGD) of the selectivity filter (SF), which is located within the pore domain and is highly conserved across potassium channels found in eukaryotic and prokaryotic organisms (Labro & Snyder, 2012) (Figure 1). As shown from the crystal structure of KcsA channel, this highly conserved sequence provides four

binding sites for potassium ions, which can be coordinated from the carbonyl oxygens of the TVGYG residues (Doyle et al., 1998). These oxygen atoms have a precise geometry that allows the SF to snugly fit potassium, but not sodium ions. The resulting unique structure is supported by a network of interactions among amino acid residues located around the SF, as shown for the KscA and KirBac1.1 channel (Cheng, McCoy, Thompson, Nichols, & Nimigean, 2011; Wang et al., 2019). This network provides a scaffold that is essential to keep the geometry of the SF in place.

Figure 1 Similarities among selectivity filter (SF) of potassium channels from different kingdoms of life. (A) The SF sequence (TVGYGD) is highly conserved across different species. The amino acids encoding for the SF and the 373 position are highlighted in orange and green, respectively. (B) High structural similarity among three structures of different SFs from bacterial and mammalian species.

Besides providing high selectivity for potassium ions, the TVGYGD sequence allows maximum conductance by bringing the energy cost to place potassium ions within the SF close to zero. Interestingly, single-channel conductance varies across potassium channels (from 5 up to 270 pS), and cannot be explained from differences in the amino acid sequence or structure of the SF (Morais-Cabral, Zhou, & MacKinnon, 2001). Different studies have found that residues located at the inner side of the pore partially determine single-channel conductance. In the case of BK channels, the high single-channel conductance can be partly explained by the presence of negatively charged residues (*i.e.*, glutamates), which attract cations at the entrance of the SF (Brelidze & Magleby, 2005). Moreover, a wide inner pore entrance has been shown to provide a low-resistance pathway for potassium ions to reach the SF (Geng, Niu, & Magleby, 2011). Two examples are the Shaker and Kv1.2/2.1 channels, whose low single-channel conductance correlates

well with a narrow and hydrophobic internal pore (del Camino, Kanevsky, & Yellen, 2005; Long, Campbell, & Mackinnon, 2005).

Other studies have also shown that not only residues at the inner side of the pore, but also at its outer side influence single-channel conductance. In the case of Shaker, single-channel conductance decreased upon mutating residues that are located at the exit of the SF (MacKinnon & Yellen, 1990). Similarly, we have observed a decrease in single-channel conductance, when we characterized the effect of the SCA19/22 mutation M373I (Chapter 2). In another case, single-channel conductance was increased from the substitution of the lysine at position 356 with glycine in Kv2.1 (Trapani, Andalib, Consiglio, & Korn, 2006). However, in BK channels, substitution of residues at the exit of the pore have little effect on single-channel conductance (Carvacho et al., 2008). Whether the single-channel conductance of the Kv4.3 channel can be increased by introducing a hydrophilic amino acid at the exit of the pore, as in the case of Kv2.1, remains to be investigated.

In this chapter, we aim to study the effect of a hydrophilic substitution (M373E), located in the SF, on the single-channel conductance of the Kv4.3 channel. We already know that a hydrophobic substitution (*i.e.*, M373I) results in the reduction of permeation events for potassium ions, and hypothesized that the hydrophobicity of position 373 inversely correlates with single-channel conductance of the Kv4.3 channel. Here, we showed that mutating M373 into a hydrophilic amino acid (*i.e.*, glutamic acid) increased the permeation of potassium ions by increasing the rehydration efficiency of potassium at the pore exit. This work confirms our hypothesis and further supports the role of residues at the exit of the SF in affecting single-channel conductance, not only in Shaker and Kv2.1, but also in the Kv4.3 channel.

2 Results

2.1 *The M373E mutant channel does not generate whole-cell currents*

The M373E mutant channel was designed *in silico* and, up to date, there is no report of cases carrying this variation in GnoMAD (assessed October 2019). We first checked whether the M373E mutant channel is expressed at PM and generates current. Therefore, we transiently expressed the M373E mutant channel in Chinese Hamster Ovary (CHO) cells and performed electrophysiological recordings in the whole-cell configuration. Cells were stimulated by commanding the membrane potential to +60 mV from a holding potential of -90 mV in 10 mV increments. The M373E mutant channel did not generate any ionic currents, likely caused by

retention within the endoplasmic reticulum (ER) and absence at the PM (Figure 2A-B).

Figure 2 Activity and localization of the M373E mutant channel. (A) CHO cells transiently expressing the M373E mutant channel in the presence and absence of KChIP2b did not generate any detectable current, as shown by the representative traces of whole-cell recordings. Whole-cell recordings are also shown for CHO cells expressing the WT channel in the presence of KChIP2b and untransfected cells. The whole-cell currents were elicited as described in the text by depolarizing the membrane potential from -90 mV up to +60 mV in 10 mV steps from a holding potential of -90 mV. (B) The M373E mutant channel is retained, when expressed in HeLa cells; KChIP2b partially rescues the retained phenotype. HeLa transiently expressing the M373E mutant channel in the absence and presence of KChIP2b-Emerald were stained using specific antibody against Kv4.3 (green/red) and calnexin (red). Nuclei were stained with DAPI (blue). Scale bar 10 μ m.

Previous studies have shown that co-expression of the auxiliary subunit KChIP2b enhances the whole-cell currents of Kv4 channels by increasing the expression of channel at the PM (Bähring et al., 2001; Wollberg &

Bähring, 2016). Thus, we co-expressed KChIP2b to enhance the surface expression of the M373E mutant channel in an attempt to get the M373E mutant channel to the PM and measure whole cell currents using whole-cell electrophysiology recordings. We found that co-expression of KChIP2b slightly increased the presence of the M373E mutant channel at the PM in HeLa cells (Figure 2B). However, in CHO cells transiently expressing the M373E channel and KChIP2b we were still unable to measure currents, whereas, we were able to record whole-cell currents from CHO cells expressing the WT channel in the presence of KChIP2b (Figure 2A-B). Our data suggests that the M373E mutant channel does not generate any current as a result of partial ER retention and/or reduced channel activity.

2.2 *The M373E mutant channel is functional, but generates small whole-cell currents compared to WT*

In a second attempt to enhance the surface expression of the M373E mutant channel, we expressed the Kv4.3 channel at low temperature (*i.e.*, 30 °C) facilitating refolding of the protein as described before (Duarri et al., 2015). Under these conditions, the M373E mutant channel generated whole-cell currents, which resembled fast activating and inactivating currents, typically observed for the WT Kv4.3 channel (Figure 3A). Compared to WT, the whole-cell conductance of the M373E mutant channel was significantly lower; 33.2 ± 15.0 nS ($n = 7$), compared to 3.1 ± 1.0 nS ($n = 5$), respectively (Figure 3B). This data shows that the M373E mutant channel generates currents, but the change of M373 to E significantly reduced whole-cell conductance.

Next, we wanted to investigate whether the M373E variation has effect on the activation voltage of the channel. When compared to the normalized peak currents of WT, in the presence of KChIP2b, the M373E mutant channel generated whole-cell currents at -50 mV, instead of -40 mV. Moreover, the whole-cell currents generated by the M373E mutant channel were not rectified by voltages at positive membrane potentials (from +20 mV to +60 mV) (Figure 3C). This suggests that, once at the PM and in its open state, the M373E mutant channel may be more conductive than the WT channel.

Figure 3 The M373E mutant channel activity at whole-cell and single-channel level. (A) CHO cells expressing the M373E mutant channel generate fast activating and inactivating currents in the presence of KChIP2b. The sweep elicited by depolarizing the membrane potential to -50 mV is highlighted in red. (B, C) The M373E mutant channel generates less whole-cell currents and activates at more hyperpolarized membrane potentials compared to WT. Whole-cell conductance and normalized currents are shown for the WT and M373E mutant channels expressed in different conditions (see legend in panel B). (D) Single-channel currents generated by the M373E mutant channel are higher compared to WT and M373I mutant channels. Sample traces show single-channel currents for the WT, M373I (reproduced from Chapter 2), and M373E mutant channels recorded at +60 mV. Different open level are marked using dashed lines. The whole-cell currents were elicited by stepping the membrane potential from -90 mV up to +60 mV in 10 mV step for 2 s. The single-channel currents are elicited by stepping the membrane potential to +60 mV for 1 min. The membrane potential is hold at -90 mV for both the whole-cell and single-channel recordings.

2.3 M373E mutation increases WT single-channel conductance

We next aimed to investigate the single-channel conductance of the M373E channels at 30 °C in the cell-attached configuration. Single-channel currents were recorded as short transient openings, as shown in Figure 2D. We used these currents to estimate the single-channel conductance of the M373E mutant channel and compared the outcome to estimate the single-channel conductance of the M373I mutant and the WT channels. We observed that the M373E mutant channel conducted 2.3-time and 2.4-time more compared to WT and M373I mutant channel (WT + KChIP2b: 9.6 ± 2.3 pS, $n = 6$; M373I + KChIP2b: 9.3 ± 0.5 pS, $n = 4$; M373E + KChIP2b: 22.2 ± 4.3 pS, $n = 5$).

Table 1 Number of permeation events of potassium ions and single-channel conductance for WT, M373I, and M373E mutant channels estimated from MD simulations and electrophysiological measurements at the single-channel level.

Kv4.3	Permeation events	Conductance in simulation (pS)	Conductance in experiments (pS)
WT	45	7.1	9.6 ± 2.3 (n = 6)
M373E	70	11.3	22.2 ± 4.3 (n = 5)
M373I ¹	24	3.8	9.3 ± 0.5 (n = 4)

¹: the M373I single-channel conductance has already been reported in Chapter 2.

2.4 The M373E mutant channel shows more permeation event compared to the WT and M373I mutant channel in MD simulations

We validated our electrophysiological observations by measuring the number of permeation events and single-channel conductance using MD simulations for the WT, M373I, and M373E mutant channels. We observed that both the permeation events and the single-channel conductance of the M373I and M373E mutant channels are lower and higher compared to WT, respectively (WT: p.e. 45, c. 7.1 pS; M373I: p.e. 24, c. 3.8 pS; M373E: p.e. 70, c. 11.3 pS). This corroborates our experimental data regarding single-channel conductance and validates the MD model for predicting the effect of mutations on channel function (Figure 3D, Table 1).

2.5 The M373E variation does not affect the pore radius, but increases the hydration of the channel pore

We examined the effect of the M373E variation on the structure of the Kv4.3 channel *in silico* in order to pinpoint the molecular determinant which leads to the increase of single-channel conductance. Since the variant is located at the exit of the pore, we generated a pore profile and examined the average pore radius over the first 350 ns of each simulation for WT, M373I, and M373E mutant channels embedded in a POPC lipid bilayer and subjected to an applied voltage of 1 V. Our results showed that the radius of the pore for the WT, M373I, and M373E mutant channels are similar to each other (Figure 4A). The MD simulations suggest that both the M373I mutation and the M373E variant do not modulate single-channel conductance by affecting the pore radius of the channel.

Next, we examined the hydration of the pore for the WT, M373I, and M373E mutant channels using MD simulations. We hypothesized that a

fully hydrated pore, generated by the presence of hydrophilic residues (glutamic acid, E), facilitates the passage of potassium ions from the SF into the extracellular environment compared to a less hydrated pore, thus resulting in an increase of single-channel conductance. We found that potassium ions are more hydrated in the M373E mutant channel compared to the WT and the M373I mutant (Figure 4B). This data suggests that the single-channel conductance was affected by the hydration state, and not by the radius of the channel pore.

Figure 4 Average pore radius, water coordination, and average number of contacts for the WT, M373I and M373E mutant channels. (A) The pore radius profiles were overall similar among the WT (red), M373E (cyan), and M373I (blue) mutant channels, but the SF of the M373E and M373I mutant channels is more and less constricted compared to WT. (B) Potassium ions are more hydrated in the M373E mutant channels compared to WT and M373I mutant channels. Water coordination number and distribution of potassium ions of WT, M373I, and M373E mutant channels are shown as dashed and solid lines. (C) The M373E mutant channel has generally more contacts with hydrophilic residues compared to the M373I mutant channel. The average number of contacts for residue 373 with selected residues 2 is shown for WT, M373I, and M373E mutant channels. The average number of contacts was measured by averaging the total number of contacts over the four chains and it was normalized dividing by the total number of frames from the whole trajectory. The cutoff for considering a residue in contact with residue 373 was 3.0 Å. The Kv4.3 was embedded in a POPC lipid bilayer and simulated with an applied voltage of 1 V. The analysis was performed over the whole trajectory.

2.6 The glutamic acid amino acid at position 373 has more contact with hydrophilic amino acids compared to isoleucine or methionine at the same position

According to Wimley and White hydrophobicity scale of amino acids, the glutamic acid (E) and isoleucine (I) will have more and less contact with water and other hydrophilic amino acids, respectively (Wimley & White, 1996). Contacts with other hydrophilic/hydrophobic residues will further amplify the hydration/dehydration of the channel pore by attracting or repelling water towards the exit of the SF. Therefore, we examined the nature of contacts between the amino acids around the pore domain in close proximity of the SF (residues 350-375) for the WT, M373I, and M373E mutant channels.

We found that M373E mutant channel had less contacts with residues 350-373 compared to both WT and M373I. Generally, E at position 373 exhibited fewer contacts with surrounding hydrophobic residues compared to M and I at the same position, whereas more contacts were observed with the hydrophilic lysine (K) at position 350 compared to M. I at position 373 had no contact with residues 321-349 and 376-402, but exhibited on average more contacts to residues 350-375 compared to M and E at the same position. In particular, we observed more contacts with residues K350, F351, V363, G369, and G371 compared to the M residue 373 (Figure 4C). The M373E variant and M373I mutation generally resulted in an increase and decrease of contacts with neighboring hydrophilic and hydrophobic residues, respectively. Overall, our MD data showed that the M373E mutant channel has a higher single-channel conductance compared to WT and the M373I mutant channel, due to an increased hydration of the channel pore.

3 Discussion

In this chapter, we investigated how methionine at position 373 affects single-channel conductance of the WT Kv4.3 channel. In Chapter 2, we showed that a hydrophobic substitution (M373I) leads to a reduction in the single-channel conductance, whereas a hydrophilic substitution (M373E) has the opposite effect (this chapter). This change in single-channel conductance results from an alteration in the hydration of the channel pore, not from differences in the pore radius. Moreover, substitutions at position 373 alter the nature of contact between this residue and the neighboring ones. Overall, this work proves how the hydrophobic nature of amino acids located at the exit of the pore modulates single-channel conductance.

We showed that the M373I mutation and M373E variant affect single-channel conductance by reducing and increasing the hydration of the pore domain at the exit of the SF also known as hydrophobic gating (Rao, Klesse, Stansfeld, Tucker, & Sansom, 2019). This suggests that, in the presence of a hydrophobic residue such as I, the SF is open, but dewetted, therefore potassium ions move less efficiently through the water-deprived cavity. On the contrary, in the presence of a hydrophilic residue such as E, the opposite seems to happen; a fully hydrated SF facilitates the passage of potassium ions leading to an increase in single-channel conductance. Hydrophobic gating differs from other type of gating, where the passage of ions is physically blocked from the presence of a protein domain (e.g., N-terminal), an amino acid (e.g., G371R), or a molecule (e.g., toxin). Several studies have already reported hydrophobic gating in different ion channels, but this phenomenon was not yet reported for Kv4.3. The presence of a hydrophobic gate is well established in the mouse serotonin 5-HT₃ receptor, where a hydrophobic barrier, located at the bottom of the pore, blocks the passage of ions (Hassaine et al., 2014; Trick et al., 2016). In BK channels, dewetting of the internal cavity, located below the SF, resulted in a complete loss of ion permeation (Jia, Yazdani, Zhang, Cui, & Chen, 2018). Similarly, dewetting of the pore has been observed in K2P and Kv1.2 potassium channels that also coincided with direct loss of ion conductance, (Aryal, Abd-Wahab, Bucci, Sansom, & Tucker, 2014; Jensen et al., 2010). Our study confirms the idea that voltage-gated potassium channels (Kvs) possess a hydrophobic gate and, in the Kv4.3 channel, this gate can be altered by mutations at position 373.

Several studies have shown that amino acids, located at the bottom of the pore, affect single-channel conductance in Kv channels and have proposed that the determinant of single-channel conductance lies at the inner side of the pore (Naranjo, Moldenhauer, Pincuntureo, & Díaz-Franulic, 2016). However, other studies have shown how mutations at the exit of the SF can also modify single-channel conductance (MacKinnon & Yellen, 1990; Trapani et al., 2006). Based on our results we suggest that residues located at the exit of the pore, can also be determinants of single-channel conductance. We put forward the idea that several amino acids located at both the inner and outer side contribute to the modulation of single-channel conductance.

This work also showed that, unlike M373I, the M373E variant caused severe retention of Kv4 channel within the endoplasmic reticulum in the absence of KChIP2b. While KChIP2b co-expression was enough facilitate

M373I mutant channel trafficking to the membrane, for the M373E mutant channel this was not enough and the cells expressing the mutant channel had to be cultured at lower temperatures to enhance trafficking to the PM. Previously, other SCA19/22 mutants (*i.e.*, Δ F227, T352P, M373I and S390N) have been reported to have similar effects (Duarri et al., 2012). These studies suggest that the residue at position 373 plays an important role in protein folding and stability.

Overall, we showed the importance of hydration of the channel pore in modulating single-channel conductance of the Kv4.3 channel. This highlights the values of using the *in silico* Kv4.3 model to predict the effect of genetic variations that may be or may not be linked to disease.

4 Material and methods

4.1 Molecular biology

The WT *KCND3S* cDNA (NM_172198) was obtained from a previous study and the M373E variation was introduced using site directed mutagenesis (Duarri et al., 2012). The mutant *KCND3* cDNA was subcloned into the pcDNA3.1. The rat KChIP2b was also obtained from previous work (Duarri et al., 2012). pmaxGFPTM (Lonza) or pEGFP-N1 (Clontech) were used to follow transfected cells. Chinese Hamster Ovary (CHO) cells (Sigma Aldrich) were chosen as the expression system because these cells do not express endogenous voltage-gated ion channels (Gamper, Stockand, & Shapiro, 2005). CHO cells were maintained and grown as previously described with some modifications. In summary, we used Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 medium (Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin streptomycin (Sigma Aldrich) (Duarri et al., 2015). Cells were seeded with a density of 40.000-60.000 cells/well on a 12mm diameter glass coverslip (Thermo Fisher Scientific) and were transfected after ~24 hours with two (pcDNA3.1-KCND3S and pmaxGFP/pEGFP-N1) or three plasmids (pcDNA3.1-KCND3S, pcDNA3.1-KChIP2b, and pmaxGFP/pEGFP-N1) using the Genius reagent (Westburg) according to the manufacturer instructions.

4.2 Immunocytochemistry

Immunocytochemistry was performed as described in Chapter 3.

4.3 Electrophysiology

Electrophysiological recordings were performed as described in Chapter 2. Peak currents were elicited by applying a double pulse protocol: the membrane voltage was stepped from – 90 mV up to + 60 mV with a 10 mV increment for 2 min and 200 ms. These two pulses were separated by

holding the membrane potential at -90 mV for 500 ms and 300 ms before the first (2 min) and second (200 ms) pulse, respectively. Single channel currents were elicited in the cell attached configuration by stepping the membrane voltage from resting potential (-90 mV) to different depolarized membrane voltages: $+40$ mV, $+60$ mV and $+80$ mV (which were obtained on a resting membrane potential of -10 mV, measured experimentally).

4.4 Electrophysiology data analysis

The single cell conductance analysis was performed by extrapolating peak current values at different membrane voltages (from -90 to $+60$ mV). The peak current values (from -30 mV to $+60$ mV, only for the M373E mutant from -60 mV to $+60$ mV) were fitted with a linear regression and the slope value was taken as the cell conductance. Leak current was subtracted in every single file prior any other analysis. Student's t-test was used to calculate statistical significance using MS Excel (Microsoft). Raw data was analyzed using ClampFit10 (Molecular Devices).

4.5 Kv4.3 Shal K⁺ channel system set up in the open state and molecular dynamics simulations

The model of the WT Kv4.3 channel was set up as in Chapter 2. The psfgen mutate command of VMD was employed on this starting system to generate M373I and M373E (in the pore domain (PD) next to the SF)) mutated Kv4.3 transmembrane domain (TMD) systems. The total number of atoms of each Kv4.3 TMD system, including water and ions, was about 157,000.

4.6 Molecular dynamics simulations

The MD simulations were run on the same system as in Chapter 2 with some modifications. Each system was subjected to the following protocol: (i) 5000 steps of energy minimization with the position of all protein atoms, crystallographic ions, POPC polar headgroups, water and ions fixed with a force constant of 1 kcal/mol·Å²; (ii) 25 ps and 100 ps NpT simulations with 0.5 fs and 1.0 fs timesteps, respectively, with the position of all protein atoms, crystallographic ions, POPC polar headgroups, water and ions fixed using a force constant of 1 kcal/mol·Å²; (iii) 5000 steps of energy minimization followed by 75 ps and 100 ps NpT simulations with 1.5 fs and 2.0 fs timesteps, respectively, with the position of all protein atoms and crystallographic ions fixed using a force constant of 1 kcal/mol·Å²; (iv) 5000 steps of energy minimization followed by a 10 ns NpT simulation with a 2.0 fs timestep and all protein atoms and crystallographic ions with harmonic positional restraints using a force constant of 1 kcal/mol·Å²; (v)

10 ns NpT simulation with a 2.0 fs timestep using harmonic positional restraints on all protein backbone atoms and crystallographic ions with a force constant of 1 kcal/mol-Å²; (vi) 10 ns NpT simulation with a 2.0 fs timestep and a gradual release of harmonic positional restraints on all protein backbone atoms keeping the crystallographic ions fixed with a force constant of 1 kcal/mol-Å²; (vii) 300 ns NpT equilibration with a 2.0 fs timestep, all crystallographic ions constrained with harmonic positional restraints using a force constant of 1 kcal/mol-Å² and distance harmonic restraints on opposing carbons of the carbonyl moieties of the selectivity filter with a force constant of 10 kcal/mol-Å²; (viii) 50 ns NpT equilibration with a 2.0 fs timestep and a gradual release of harmonic positional restraints and distance harmonic restraints (Starek, Freitas, Berneche, & Tobias, 2017). Then, the 350 ns equilibrated structure having 150 mM KCl was subjected to the addition of ions to reach an ionic strength I of 500 mM KCl using the CHARMM GUI Membrane builder for the calculation of the number of K⁺ and Cl⁻ ions to be added and the Autoionize plugin of VMD 1.9.3; (ix) 5000 steps of energy minimization followed by a 20 ns NpT equilibration of the new ionized structure without harmonic positional restraints and distance harmonic restraints; and (x) 300 ns NpT production run with an applied electric field generating a membrane potential of 1 V with distance harmonic restraints using force constants of 1, 0.5 and 0.25 kcal/mol-Å² on opposing carbon atoms of carbonyl groups of the selectivity filter (residues 367 to 372) for 10 ns, 10 ns and the remaining of the production run, respectively. The center of mass motion removal was employed in all simulations. All simulations of NpT equilibration runs were carried out at a constant physiological temperature of 310.15 K using a Langevin dynamics scheme and at 1 atm pressure using a Langevin-Nosé-Hoover piston method (Feller, Zhang, Pastor, & Brooks, 1995; Martyna, Tobias, & Klein, 1994). All production runs were performed at a constant physiological temperature of 310.15 K in a NVT ensemble applying a homogeneous external electric field (E_z) along the z-axis (L_z) perpendicular to the membrane proportional to a defined voltages (V) of 1 V (E_z = -V/L_z) (Chandramouli, Di Maio, Mancini, Barone, & Brancato, 2015).

4.7 Analysis of the MD trajectories

The pore radius was measured with the HOLE program (Smart, Neduvellil, Wang, Wallace, & Sansom, 1996). All sequences were aligned by performing a complete alignment in the multiple alignment mode. The number of permeation events were measured with an in-house script, which divided the simulation box in three different regions, the first one was before the intracellular side of the lipid membrane, the second one

included the selectivity filter and the third one was located after the extracellular side of the lipid membrane in the bulk solution. The first and last two regions were interspaced by buffer regions spanning the areas occupied by the cavity and the interface between the selectivity filter and the bulk solution, respectively. The conductance, C , of K^+ ions, with units in Siemens ($S = 1A/1V$) defined as the number of electron charges per nanosecond, was calculated by using the conversion factor of $160.217 \times N_e / ns$, corresponding to 1 pA, and the following equation: $C = 160.217 \times \text{Number of permeation events/time of simulation in ns}$. The water coordination number and the distribution of K^+ ions were estimated using in-house codes. The analysis of contact maps and distances were performed with Gromacs 5.0.7 built-in analytical tools. Images, movies and trajectory analyses were performed using graphical tools, analytical plugins and Tcl scripts in VMD 1.9.3 (Humphrey, Dalke, & Schulten, 1996).

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Chapter 6

An *in vitro* platform for the characterization of the human voltage-gated potassium channel Kv4.3 from a bottom-up perspective

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Action potentials mediate the communication across neuronal cells. These action potentials in repetitively firing neurons are modulated by the fast-transient A-type potassium current, which takes part in regulating the spike timing, firing frequency, and backpropagation of the action potentials. A-type current is encoded by a quaternary complex comprised of the ion conducting Kv4 channel and its regulatory partners including different cytosolic proteins and lipids. How do some of these components interact with the Kv4.3 channel and fine-tune its activity is not known as their interaction cannot be readily studied in common cellular expression systems due to intrinsic limitations posed by the cells, e.g., lack of control over the expression of cellular components, low signal to noise ratios in spectroscopic techniques caused by cellular components. In order to address this problem, we reconstituted the heterologously expressed and purified Kv4.3 channel into synthetic lipid bilayers. Our data show that the single-channel activity of the reconstituted Kv4.3 resembles its activity in mammalian cells and does not need cellular components for its functioning. Thus, the reconstituted channel provides a new platform that is compatible with many biophysical techniques for detailed structure-function studies of Kv4.3 in a highly-controlled environment.

1 Introduction

The A-type potassium current is a fast, transient outward potassium current occurring in cardiomyocytes and neurons. It is generated *in vivo* by various combinations of different molecular components: three different members of the Kv4 channel subfamily, two types of auxiliary proteins, one type of accessory proteins, and different membrane lipids. While the Kv4 channel protein is solely responsible for ion conduction, the other components fine-tune the A-type potassium current by modulating peak current, ion conductance, and channel gating properties. The Kv4 channel subunits come together to form a heterotetrameric complex (Figure 1) (Pioletti, Findeisen, Hura, & Minor, 2006; Huayi Wang et al., 2007). The auxiliary and accessory proteins voltage-gated potassium channel beta (Kv β) and Kv channel-interacting protein (KChIP) bind to the channel from the cytoplasmic side. The dipeptidyl peptidase-like protein (DPLP) has been proposed to interact with the voltage-sensing domain of the Kv channel in the membrane via one single transmembrane domain. Additionally, DPLP might have a cytoplasmic end that interacts with the Kv channel from its cytoplasmic side (Figure 1). The resulting quaternary complex activates at subthreshold potentials (e.g., -60 mV), and rapidly inactivates to generate the A-type current. The Kv4 channel complex becomes inactive at membrane potentials more positive than -50 mV until the membrane is hyperpolarized. In neurons, the specific voltage sensitivity and the kinetic properties of Kv4 channel define the physiological role of the A-type current, which is regulating the spike timing, firing frequency, and backpropagation of action potential in repetitively firing neurons (Connor & Stevens, 1971; Kim, Wei, & Hoffman, 2005; Shibata et al., 2000).

The auxiliary and accessory proteins shape the activation and inactivation kinetics of the A-type potassium current depending on the tissue type and intra- or extracellular signals. When heterologously expressed in HEK293 cells, Kv β 2 has been shown to increase the peak current density and expression at the plasma membrane of the Kv4.3 channel (Yang, Alvira, Levitan, & Takimoto, 2001). Protein Kinase C (PKC) has been shown to either reduce or increase channel inactivation for the Kv4.3 short and long isoform, respectively (Xie, Bondarenko, Morales, & Strauss, 2009). KChIPs affect the electric properties and assist the folding of Kv4.3. KChIP1, KChIP2, and KChIP3 increase the surface expression of the wild-type and pathogenic channel. KChIP1 and KChIP2 also slow down the inactivation and speed up the recovery from inactivation (Bourdeau, Laplante, Laurent, & Lacaille, 2011; Cui, Liang, & Wang, 2008; Duarri et

al., 2012; Patel, Campbell, Morales, & Strauss, 2002; Patel, Parai, Parai, & Campbell, 2004; Takimoto, Yang, & Conforti, 2002). KChIP4a has been shown to function as a surface expression suppressor and to promote channel inactivation (Liang et al., 2009; Tang et al., 2013). DPLPs enhance the trafficking of Kv4 channel to the plasma membrane and accelerate the kinetics of inactivation and recovery from inactivation. Moreover, DPLPs also increase single-channel conductance (Henry H. Jerng, Lauver, & Pfaffinger, 2007; Henry H. Jerng, Qian, & Pfaffinger, 2004; Kaulin et al., 2009; Lin, Long, Hatch, & Hoffman, 2014; Seikel & Trimmer, 2009; Sun et al., 2011; Zagha et al., 2005).

Figure 1 The quaternary Kv4 channel complex. Only two out of four subunits are represented as embedded into a lipid bilayer (lipid heads and acyl chains are shown as grey balls and black lines, respectively). The different protein domains are highlighted: VSD (red), S4/S5 linker (dark blue), pore domain (green), and N-terminal domain (orange line). KChIP (magenta) is depicted in its bound form, that is immobilizing the N-terminal domain of the Kv4 channel. DPLP (cyan) is shown with its putative N-terminal domain inserted within the lipid bilayer. Kvβ (ochre) is shown in a hypothetical bound state based on the model by Pioletti et al. 2006.

In addition to the proteins, lipids are also important for regulating the functioning of voltage-gated ion channels, including Kv4. Phosphatidylinositol 4,5-bisphosphate (PIP₂), for example, is a membrane phospholipid that is essential for the conduction of ions in Kv7.2-7.3 channels (Brown, Hughes, Marsh, & Tinker, 2007). In Kv1.2, the absence of PIP₂ results in a decrease of the current amplitude, and it shifts the channel activation to more hyperpolarized membrane voltages (Rodriguez-Menchaca et al., 2012). The role of PIP₂ is less established, and it is a matter of debate for other ion channels, such as Kv1.1, Kv1.4, and Kv2.1 (Delgado-Ramírez et al., 2018; Kruse, Hammond, & Hille, 2012; Oliver et al., 2004). Polyunsaturated fatty acids (PUFAs) are known to affect the Kv4 channel activity. For instance, arachidonic acid

suppresses peak currents and increases the inactivation rate of the Kv4 channel in a KChIP-independent manner (Boland, Drzewiecki, Timoney, & Casey, 2009; M H Holmqvist et al., 2001; Villarroel & Schwarz, 1996).

All previous studies show how the A-type potassium current is the result of a complex interaction between different cellular components. These studies are performed in cellular systems (e.g., cell lines and brain slices), where the electrical activity of the Kv4 channel complex has been recorded using different electrophysiological assays (e.g., patch clamp and two electrode voltage-clamp). Although this approach works to study the native A-type potassium current, it has some limitations for studying the effect of SCA19/22 mutations, auxiliary proteins or lipids on the Kv4.3 channel structure and function at the molecular level. First, the approach does not always work, because the Kv4.3 channel, which carries a SCA19/22 mutation, may not localize at the plasma membrane, but is retained within the intracellular compartments (Duarri et al., 2012). This leaves the experimenter unable to test the functionality of the SCA19/22-mutated Kv4.3 channel using the classical patch clamp techniques (*i.e.*, whole-cell, inside-out, outside-out) that records channel activity from the plasma membrane. Second, the presence of endogenous cellular components cannot be controlled using the expression systems as mentioned above. Different cellular systems express endogenous KChIPs, which are known to differentially regulate the Kv4.3 channel activity (Patel et al., 2002). Last, it is not always possible to study the effect of integral membrane lipids due to limited control over the expression systems. However, these three limitations can be overcome. First, non-classical patch clamp techniques (*i.e.*, intracellular patch technique and organelle membrane derived patch clamp) can be used to record channels, which are retained in the intracellular membrane. Second, classical molecular biology techniques can be employed to regulate the expression of endogenous regulatory proteins (Jonas, Knox, & Kaczmarek, 1997; Shapovalov et al., 2017; Sorgato, Keller, & Stühmer, 1987). Third, inhibitors (e.g., wortmannin) can be applied to the cell system in order to stop the synthesis of specific lipids (e.g., PIP₂). However, there are several unknown secondary effects generated by the manipulation of living systems using genetic tools and drugs. Therefore, in order to overcome all these three limitations at once, the Kv4.3 channel could be studied in cell-mimics, such as liposomes or planar lipid bilayers.

Based on this need and inspired by the work of others, who have established protocols to study different ion channels (e.g., MscL, Kv1.3, Kv7.1, Slo2.1, and KcsA) in biochemically defined lipid systems, we set

out to establish an *in vitro* platform for studying the Kv4.3 channel complex (Garten, Aimon, Bassereau, & Toombes, 2015; Klaerke et al., 2018; Koçer, Walko, & Feringa, 2007; Spencer et al., 1997).

In this chapter, we present a highly-controlled platform to study the wild-type Kv4.3 channel complex that would allow building A-type current in a bottom-up fashion by incorporating individual components of the quaternary channel complex one at a time into artificial lipid bilayers. The platform is built in three steps. First, we tested the functionality of the Kv4.3 channel in the absence of cytosolic components (e.g., auxiliary proteins). Second, we investigated the feasibility of Kv4.3 channel production in a simple heterologous expression system and its isolation from the host membrane, e.g., the yeast *Pichia* expression system. Third, we reconstituted the Kv4.3 channel into a brain-like lipid environment and validated its activity. Overall, we showed that the Kv4.3 channel could be reconstituted into a lipid bilayer *in vitro*, where it is functional. This highly-controlled platform can be used to study the biophysical properties of the A-type potassium current in a reductionist fashion. Different components (e.g., native and pathogenic Kv4.3 channels, auxiliary proteins, and specific lipids) constituting the A-type potassium current can be modified, incorporated or removed from the system individually, so the effect of each component can be separately studied in a highly controlled manner. This will help to gain insight both on the fundamental working mechanism of the Kv4.3 ion channel as well as on the contribution of different biological components on generating the A-type potassium current *in vivo*.

2 Results

2.1 *The Kv4.3 channel is functional in the absence of cytosolic components*

Since the goal of this study is to establish an *in vitro* functional assay, where the Kv4.3 channel can be characterized outside the cell, we first checked whether the cytosolic components are necessary for the proper functioning of the Kv4.3 channel. This was achieved by recording Kv4.3 single-channel currents in the cell-attached and the inside-out patch clamp configuration in Chinese hamster ovary (CHO) cells as shown in Figure 2A. In the first configuration, the cell remains intact, and the cytosolic components are still in contact with the cell membrane, where the Kv4.3

channel is located. In the second configuration, a piece of membrane is excised from the cell, and the channel is not in contact with any cytosolic

components anymore and exposed to the external buffer instead. In both cases, the channels were activated by commanding the membrane potential from a hyperpolarized resting state (*i.e.*, -90 mV) to different positive membrane voltages (+40, +60, +80 mV for the cell-attached recordings and +50 mV, +60 mV, +70 mV for the inside-out recordings).

Figure 2 Wild-type Kv4.3 single channel currents and conductance. (A) Schematic representation of a patch clamp setup (left). One ion channel (grey) is depicted embedded into a lipid bilayer. The potassium ions (red) are also shown moving across the channel. Patch pipette and amplifier are depicted together with an exemplary trace showing single channel activity. (B) The cell attached (middle) and inside out (right) patch clamp configurations. The sealed patch is highlighted in red and the cytosolic environment is shown in white. (C, D) A sample trace of a cell attached and inside out recording. (top) Overview of the recording is shown. Several events can be seen along the entire length of the trace. (middle) Enlarged view of a section of the recording. Here, the single channel events (O1 and O2) can be distinguished. (E, F) Single channel currents versus different voltages from several patches in the cell attached and the outside out configurations, respectively. The estimated or theoretical reversal potential is also plotted: -10 and 0 mV.

The currents are fitted with linear regression and the slope represents the theoretical unitary conductance in nS. (C: closed, O: open).

As can be seen in Figure 2B and C, the same single-channel currents are observed when the channels were exposed to the cellular environment and the external buffer. This means that the Kv4.3 channel activity does not require any cytosolic components to be functional. Furthermore, the single-channel conductance is the same in the presence and absence of the cytosolic components: 15.1 ± 1.2 pS and 14.5 ± 2.3 pS for the cell-attached and inside-out patch, respectively (Figure 2D, E). Taken together these results show that Kv4.3 requires only the lipid bilayer for its functioning and can theoretically be studied *in vitro* in artificial lipid bilayers.

2.2 Expression and isolation of the Kv4.3 channel

Knowing that Kv4.3 channel is functional outside the cellular environment, we established a protocol for the production and the isolation of Kv4.3, which can be then used to generate an *in vitro* reconstituted system. We chose the *Pichia pastoris* (*P. pastoris*) expression system, since it is a simple host that has been proven successful for the production of several eukaryotic proteins, including membrane proteins (Bertheleme, Singh, Dowell, & Byrne, 2015; Egawa et al., 2009; Weiss, Haase, Michel, & Reiländer, 1995). Specifically, the *Pichia* system has two advantages. First, it can perform post-translational modifications, which are essential for the proper functioning of eukaryotic proteins. Second, it is a system which is easy to handle and cost-effective.

A C-terminally His-tagged full-length Kv4.3 cDNA was cloned in the pPICZ plasmid, which was then stably integrated into the *P. pastoris* genome for methanol-inducible expression of Kv4.3 channel protein. Different parameters can be optimized in order to maximize protein yield, namely the methanol concentration, induction time and duration, and medium composition. We tested three different methanol concentrations (0.32%, 0.46%, and 0.5%), several induction periods (14 h, 24 h and 46 h) and three types of medium compositions (unbuffered, pH-buffered and yeast extract/peptone-supplemented) in small-scale experiments (10 mL growth medium). None of these parameters resulted in a significantly increased protein yield (data not shown). Therefore, we chose to grow the *Pichia* strain in a pH-buffered medium in order to avoid acidification of the medium which inhibits cell growth. Based on these optimization experiments, we set up a protocol, where the Kv4.3 expression was induced using 0.5% methanol for 24 h at pH 6.0.

Figure 3 The purified Kv4.3 channel. The elution profile (6 elution fractions) is shown for the purification of the Kv4.3 (A) and the mock sample (B). Three protein populations are observed: two are impurities (IM1 and IM2) with a molecular weight of ~120 and ~100 kDa, one is the Kv4.3 protein with a molecular weight of ~70 kDa. The samples were run on SDS-PAGE. The gel was stained using Coomassie blue for the visualization of proteins, or the proteins were transferred onto a PVDF membrane which was stained using an antibody against Kv4.3. (C) The Kv4.3 protein has also been identified using mass spectrometry on the excised gel band which corresponds to the Kv4.3 channel. The amino acid sequence of the Kv4.3 protein is shown. The 9 peptides which uniquely matched the Kv4.3 sequence are highlighted in bold and grey. Two types of posttranslational modification are also reported.

Once the Kv4.3 expression was optimized, we isolated the channel from the membrane fraction of the yeast cells by detergent solubilization of the membrane. After testing *n*-dodecyl- β -D-maltopyranoside (DDM) and Triton X-100 as mild zwitterionic detergents for their efficiency of solubilization of the membrane, we chose Triton X100 to go further.

Next, we purified Kv4.3 from detergent-solubilized membrane fractions by immobilized metal affinity chromatography using Ni-NTA agarose matrix. We followed a standard procedure as the starting point in which, detergent-solubilized membrane fractions were incubated with the equilibrated nickel matrix for the histidine-tag to bind to the nickel. The unbound proteins were separated from the column-interacting fraction by pouring the matrix into a column and let the sample flow through the

column by gravity. Weakly bound proteins were washed away using a low concentration of imidazole in high salt buffer (1% Triton™ X-100, 50 mM Tris-HCl pH 8.0, 300 mM NaCl, 10 mM imidazole, 50 μ M ZnSO₄). We collected proteins that strongly bound to the column by washing the column with saturating concentration of histidine (235 mM) in 500 μ L elution fractions.

The presence of Kv4.3 was shown in Western blot analysis of the eluted fractions using an antibody against Kv4.3 (Figure 3A). The elution fractions obtained from this standard purification contained two other proteins as impurities that segregated in the SDS-PAGE gel at ~100 and ~120 kDa. In Western blots, these two proteins did not react to the specific antibody against Kv4.3.

In order to know whether the impurities co-elute with Kv4.3 because they are specifically interacting with the Kv4.3 or nonspecifically copurifying with it, we run the same purification procedure on a mock sample. In this case, the same production and isolation procedures were performed with cells that do not express Kv4.3. As expected, the Kv4.3 was not detected in the mock sample. However, we found the same impurities as in the Kv4.3 sample (Figure 3B), showing that they are intrinsic to the yeast cell and have no specific interaction with the Kv4.3.

The presence of Kv4.3 was also confirmed using mass spectroscopy. The protein band corresponding to Kv4.3 in SDS-PAGE gel was cut out of the gel and digested using trypsin in order to generate small peptides, which were then separated from each other based on their mass to charge ratio using LC-ESI-MS/MS (tandem mass spectrometry coupled with electrospray ionization and liquid chromatography). From the band corresponding to the molecular weight of Kv4.3 protein, nine unique peptides matched the amino acid sequence of the Kv4.3 protein and none of the peptides from the impurity bands matched the Kv4.3 protein sequence (Figure 3C).

Overall, our results show that we can produce Kv4.3 channel in the *Pichia* expression system and purify it from the host membrane. However, some impurities are present in the purified sample, and the procedure should be further optimized.

2.3 Isolated Kv4.3 is functional when embedded in a lipid bilayer

Next, we reconstituted the detergent-solubilized Kv4.3 into liposomes composed of a commercially available lipid extract (*i.e.*, total brain lipids mixture) using established methods (Koçer et al., 2007). After

reconstitution, we did SDS-PAGE and Western blot to prove the incorporation of the channel into the liposomes (Figure 4B).

In order to patch the reconstituted channels in liposomes, we converted them into giant unilamellar liposomes (GUVs) by electroformation using Nanion Vesicle Prep Pro as explained before (Birkner et al., 2012) (Figure 4A). We performed cell-attached patch on GUVs. Figure 4C shows the recorded single-channel activity from GUVs. The average single-channel current was 0.78 ± 0.04 pA at +60 mV (Figure 4A) yielding a single-channel conductance of 13.1 ± 0.6 pS, which is similar to the one measured in CHO cells, *i.e.*, 13-15 pS.

As a negative control, we patched giant liposomes with no incorporated ion channels that were prepared under the same conditions as Kv4.3. The membranes alone did not show any channel activity (Figure 4D).

Figure 4 Kv4.3 single-channel current in giant unilamellar vesicles (GUVs). (A) GUVs photographed under the microscope (scale bar = 25 μm). (B) The GUV sample contains only Kv4.3 and no other proteins. Coomassie Blue staining of GUV samples (on the left). Western blot of GUV samples using an antibody against Kv4.3 (on the right). (C, D) A representative trace of GUVs reconstituted with Kv4.3 and with only elution buffer (*i.e.*, the negative control).

Even though we could show the channel activity in liposomes, the reconstitution efficiency was not high. Having only a few channels in a

liposome is a desired situation for single-channel recordings. However, for other biophysical measurements, more channels per liposome might be needed. Therefore, reconstitution efficiency should be improved. It is well-established that the reconstitution conditions have to be tailored per ion channel. The factors affecting reconstitution efficiency, especially the lipid composition, protein to lipid ratio, the detergent of choice, and the speed of detergent removal might be optimized.

In conclusion, we showed that Kv4.3 retains its function upon isolation from a yeast membrane and reconstitution into a synthetic lipid bilayer. However, for future work requiring ensemble measurements, the reconstitution conditions need to be tailored to Kv4.3.

3 Discussion

Our work has established a platform to study the Kv4.3 channel complex in a highly-controlled fashion *in vitro*. This is achieved in two steps. First, the Kv4.3 single-channel conductance has been pinpointed to about 14 pS within the cellular environment (*i.e.*, CHO cells). This finding is corroborated by the measurement of the Kv4.3 single-channel current in two different patch clamp configurations (*i.e.*, cell-attached and inside-out). Moreover, the measurement of the single-channel conductance in two different patch clamp configurations shows that the Kv4.3 channel activity is independent of any cytosolic components (*e.g.*, KChIPs). Second, the Kv4.3 channel is reconstituted within an artificial lipid bilayer. To our knowledge, this is the first time that a voltage-gated potassium channel from the Kv4 family has been heterologously expressed, purified, and reconstituted into an artificial lipid bilayer.

Different values have been reported in the literature for the single-channel conductance of Kv4 channels (Table 1). The Kv4.1 single-channel conductance has been reported to be 5 pS (Beck, Bowlby, An, Rhodes, & Covarrubias, 2002; H H Jerng, Shahidullah, & Covarrubias, 1999). The single-channel conductance of Kv4.2 is a matter of debate; two independent studies report its value to be 4 and 18 pS (Kaulin et al., 2009; Z. Wang, Eldstrom, Jantzi, Moore, & Fedida, 2004). One value is similar to the one reported for the Kv4.1 channel, while the other is 3.6-fold higher than the one reported for the Kv4.1 channel. Similarly, two different values have also been reported for the Kv4.3 single-channel conductance: 5 pS and 18 pS (Mats H Holmqvist et al., 2002; Huizhen Wang et al., 2000). The discrepancies can be explained neither by the usage of different isoforms (*e.g.*, short and long), because the studies do not provide detailed information about the employed genes, nor by the different

species of origin, since two different values are reported for Kv4.2 and Kv4.3 channel coming from the same species. Different expression systems can also not explain the discrepancy for the Kv4.2 channel. However, the difference in the reported conductance can be explained by differences in the composition of the recording solutions used in the studies by H. Wang in 2000 and Z. Wang in 2004 (Table 1). In these studies, the resting membrane potential is not zeroed due to the asymmetric ionic composition of the recording solutions, whereas the membrane potential is zeroed in the other studies. In order to rule out any bias from differences in the ionic composition of the recording solutions, we have recorded the single-channel currents in the inside-out configuration using symmetric solution. Moreover, the studies by H. Wang and Z. Wang use a higher concentration of potassium in the pipette than the others. High potassium concentration has been shown to increase single-channel conductance (Trapani, Andalib, Consiglio, & Korn, 2006).

We have shown that the His-tagged Kv4.3 channel can be purified using affinity chromatography. This is another example, where the *Pichia pastoris* system can be used for the production of eukaryotic membrane proteins, as shown in several other studies (Hoi, Qi, Zhou, & Montemagno, 2018; Shimamura et al., 2011; Wei et al., 2017; Zhang et al., 2007). We have also shown that some impurities elute along with Kv4.3. Our data suggest that these impurities bind either to the column matrix or directly to the Nickel ion. This is also supported by another study where similar impurities are eluted, when a His-tagged protein is produced using the *P. pastoris* expression system and purified using a Nickel ion chelated by nitrilotriacetic acid (Karlsson et al., 2003). There are different ways to remove these impurities. First, the Nickel ion could be substituted with a Cobalt ion, as in (Dhillon et al., 2015). The latter gives yield to higher specificity than the former at the expense of the protein yield. Alternatively, a size-exclusion chromatography step may be added to the procedure to exclude the impurities, which have different molecular weights than Kv4.3 (Fischer et al., 2009). Other parameters that need to be optimized are solubilization conditions, such as the detergent type and concentration. These factors are crucial in order to maximize the yield for membrane protein purification (Maggioni, Hadley, von Itzstein, & Tiralongo, 2014).

Table 1 Overview of the single-channel conductance for the Kv4.1, the Kv4.2, and the Kv4.3 channels.

Species	Kv4.x	γ (pS)	Exp. system	Recording solutions	Pipette conf.	Ref.
Mouse	Kv4.1	5	<i>Xenopus</i> oocytes	Bath* – Pipette** sol.	Cell-att ached	H. H. Jerng, 1999
	Kv4.1	5	<i>Xenopus</i> oocytes	Bath* – Pipette** sol.	Cell-att ached	E. J. Beck, 2002
Rat	Kv4.2	18	HEK-293 cell line	Bath [§] – Pipette ^{§§} sol.	Cell-att ached	Z. Wang, 2004
	Kv4.2	4	tsA-201 cell line	Bath ⁺ – Pipette ⁺⁺ sol.	Cell-att ached	Y. Kaulin, 2009
n/a	Kv4.3	5	<i>Xenopus</i> oocytes	Bath* – Pipette** sol.	Cell-att ached	M. H. Holmqvist, 2002
	Kv4.3	18	<i>Xenopus</i> oocytes	Bath [†] – Pipette ^{††} sol.	Inside- out	H. Wang, 2000
Human	Kv4.3	15	CHO cell line	Symmetric sol.	Inside- out	this study

γ : single-channel conductance. Ionic composition of the recording solutions *: 130 K-aspartate, 10 KCl, 1.8 CaCl₂, and 10 HEPES, pH 7.3, adjusted with KOH. **: 96 NaCl, 2 KCl, 1.8 CaCl₂, 1 MgCl₂, 5 HEPES, pH 7.4, adjusted with NaOH. §: 135 KNO₃, 10 HEPES, 1 MgCl₂, and 1 CaCl₂, adjusted to pH 7.4 with KOH. §§: 5 KNO₃, 130 NaNO₃, 10 HEPES, 1 MgCl₂, and 1 CaCl₂, adjusted to pH 7.4 with NaOH. +: 152 KCl, 1.5 CaCl₂, 1 MgCl₂, and 10 HEPES, pH 7.4, adjusted with KOH. ++: 2 KCl, 150 NaCl, 1.5 CaCl₂, 1.5 MgCl₂, and 10 HEPES, pH 7.4, adjusted with NaOH. †: 5 NaCl, 100 KCl, 0.3 CaCl₂, 2 MgCl₂, and 10 HEPES. ††: 100 NaCl, 5 KCl, 0.3 CaCl₂, 2 MgCl₂ and 10 HEPES (pH 7.4). All reported values for the concentration of ions are in mM.

We show that the Kv4.3 channel can be reconstituted within an artificial lipid bilayer and it gives rise to currents that closely resemble the Kv4.3 currents recorded in CHO cells. These results show that voltage-sensing and gating of Kv4.3 require only the lipid bilayer. Different lipid environments are known to affect the functioning of many other ion channels. For example, lipids affect the activity of the mechanosensitive channel MscL as shown in two different studies. In the first case, when lysophosphatidylcholine is added to the lipid bilayer, the channel opens

due to changes in the distribution of pressure across the membrane bilayer (Dimitrova et al., 2016). In another case, the length of the acyl chains affects the sensitivity to pressure of the MscL channel due to differences in the hydrophobic mismatch between the MscL and the acyl chains (Perozo, Kloda, Cortes, & Martinac, 2002). As mentioned earlier, PIP₂ and PUFAs affect the channel activity of different voltage-gated ion channels. Furthermore, phosphatidic acid directly modulates the sensitivity to voltage for the Kv1.2 channel by shifting the voltage of activation towards more hyperpolarized voltages. Similarly, sphingomyelin directly modulates the voltage sensitivity of different voltage-gated channels, such as Kv and Nav (Combs, Shin, Xu, Ramu, & Lu, 2013; Hite, Butterwick, & MacKinnon, 2014). Our data suggest that the brain lipid extract is enough to reconstitute the same single-channel conductance as observed in CHO cells. However, the membrane composition of CHO cells and neurons are diverse. Several lipid species make up the CHO cell membrane: phosphatidylcholine (39%), phosphatidylethanolamine (32%), phosphatidylserine (11%), and sphingomyelin (18%) (Cezanne, Navarro, & Tocanne, 1992). In an adult human brain, the membrane composition of the gray matter is as follow: cholesterol (31.3%), phosphatidylethanolamine (19.6%), phosphatidylcholine (19.4%), phosphatidylserine (5.7%), sphingomyelin (4.2%), cerebroside (4.7%), cerebroside sulfate (1.5%), ceramide (1.6%) and other (12%) (O'Brien & Sampson, 1965). Further research is needed to establish the minimal required lipid composition of a lipid bilayer for studying the effect of individual additional lipids on the Kv4.3 channel function.

With this work, we have established for the first time a platform for studying Kv4.3 channel complex in a highly-controlled environment (*i.e.*, the artificial lipid bilayer). Kv4.3 in synthetic lipid environment would allow employing numerous biophysical and biochemical techniques that would otherwise not be feasible to use in cells (*e.g.*, as site-specific chemical labeling of the channel, various spectroscopic techniques that are disturbed by intrinsic cellular signals or components, etc.) to elucidate the working principles of the channel and molecular interactions between the channel with its individual partners and lipids at the molecular level. We have reported in Chapter 2 that the pathogenic Kv4.3 mutants not only alter the channel structure and/or function, but also affect the modulation of the channel by its partners. Therefore, a system as we generated here could be advantageous to determine the molecular interactions between the wild-type and mutant Kv4.3 channel with its modulators.

4 Methods

4.1 Plasmids

The pcDNA3.1(-)-*KCND3S*, which contains the sequence coding for the short isoform of Kv4.3 (636 amino acids, NM_172198.2), was kindly provided by Dineke Verbeek and was used for all the experiments performed in Chinese Hamster Ovary (CHO) cells. For expression of Kv4.3 in yeast, the *KCND3S* cDNA was cloned into an empty pPICZB vector (Invitrogen) with the addition of a 6xHis sequence at the C-terminus. EcoRI/XhoI restriction sites were added together with the His-tag sequence by PCR amplification of the gene using *KCND3_EcoRI_Forward* (5'-aaaaagaattcaccatggcgccggagttgcggc-3') and *KCND3_XhoI_HisSTOP_Reverse* (5'-ttttctcgagtcaatgatgatgatgatgcaaggcgagaccttgacaacatt-3') primers. The correctness of all constructs was verified by Sanger sequencing. *Escherichia coli* DH5 α was used for cloning and propagation of the plasmids.

4.2 Kv4.3-His production

The plasmid pPICZB-*KCND3S*-6xHis was transformed into the yeast *P. pastoris* SMD1168H strain (Invitrogen). The transformants were plated on selective YPDS agar (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, 1M sorbitol, 20 g/L agar) supplemented with 500 μ g/mL ZeocinTM (Invitrogen). The clone expressing Kv4.3-His (*aox1::KCND3S*-His) was selected by Western blot analysis (data not shown). For the production of Kv4.3-His, *aox1::KCND3S*-His was plated on YPD (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose) agar. A single colony was inoculated in 2 mL YPD medium. The culture was transferred into BMG medium (1.34% yeast nitrogen base, 0.00004% biotin, 1% glycerol, 100 mM potassium phosphate buffer pH 6.0) and the volume of the culture was scaled up to 0.5 L. Expression of the Kv4.3-His protein was induced by transferring the culture into BMM medium (BMG medium, containing 0.5% methanol in place of glycerol) to a final OD at 600 nm of 2.

After 24 hours of induction, the culture was pelleted at 6,000 x g for 15' at 4 °C. The pellet was squeezed through a syringe and flash-frozen in liquid nitrogen. The frozen pellet was milled for 3' at 25 Hz and cooled 30" at 5 Hz for five times using the CryoMill (Retsch). All the following steps were carried out on ice. Cell powder was resuspended in a lysis buffer (50 mM phosphate buffer pH 8.5% glycerol, 1 mM EDTA, 0.2 mM PMSF, Complete Protease Inhibitor) at 1:4 (w/v) ratio. The suspension was centrifuged at 4,000 x g for 5'. The supernatant was collected and centrifuged at 100,000 x g for 2 hours. The pellet was resuspended using

a Dounce homogenizer in 20 mL chilled lysis buffer. The resuspension was centrifuged at 100,000 x g for 30'. The pellet was homogenized in 20 mL lysis buffer supplemented with 4 M urea and centrifuged at 100,000 x g for 30'. The pellet was weighed and homogenized in 25 mM Tris-HCl pH 7.4 to a final membrane concentration of 0.7 g/mL, and saved in aliquots at -80°C for further use.

4.3 *Kv4.3-His purification*

For protein purification, the solubilization buffer (2% Triton™ X-100, 50 mM Tris-HCl pH 8.0, 300 mM NaCl, 50 μM ZnSO₄) was added to the membrane fraction with a 1:10 (w/v) ratio and the mixture was rotated for 3 hours at 4 °C. The solution was centrifuged at 100,000 x g for 1 hour. The supernatant was collected, mixed with 2 mL pre-equilibrated Ni-NTA agarose with solubilization buffer (Qiagen) and rotated for 45' at 4 °C. The mixture was poured into a 6.8 x 1.8 cm column, and the flow-through that contains unbound proteins and impurities was collected. The matrix was washed first with 10 mL washing buffer (1% Triton™ X-100, 50 mM Tris-HCl pH 8.0, 300 mM NaCl, 10 mM imidazole, 50 μM ZnSO₄) and then with 10 mL washing buffer supplemented with 40 mM imidazole. Kv4.3-His was eluted using elution buffer (1% Triton™ X-100, 50 mM Tris-HCl pH 8.0, 300 mM NaCl, 235 mM histidine, 50 μM ZnSO₄) in 500 μL aliquots. The protein concentration was determined using Bradford assay and was ~0.1mg/L of culture.

4.4 *Preparation of liposomes for Kv4.3 reconstitution*

The liposomes were prepared as previously described with slight modifications (Koçer et al., 2007). We used 1.6 mL of brain total lipid extract dissolved in chloroform (Cat. n. #131101C, Avanti Polar Lipids, Inc.). After chloroform evaporation in a rotary evaporator, the dried thin lipid film was rehydrated in 2 mL lipid buffer (10 mM Tris, 150 mM NaCl pH adjusted to 8.0 with HCl). The liposome suspension was frozen in liquid nitrogen and thawed in a 50 °C water bath for 5 times. Aliquots (500 μL) were prepared and stored at - 80 °C.

4.5 *Reconstitution of Kv4.3 into liposomes and formation of giant unilamellar vesicles*

The Kv4.3 was reconstituted into liposome as previously described with some minor differences (Koçer et al., 2007). Briefly, the liposome suspension was thawed in a 50 °C water bath. Triton-X100 (Sigma Aldrich) was added to a 1:10 volume ratio. Either the purified Kv4.3 or the elution buffer alone was added to the solution at a 1:1 (v:v) lipid to protein ratio. The solution was incubated for 30' at 50 °C. After the incubation,

200 mg (wet weight) BioBeads (SM-2 Absorbents) were added to the solution for detergent removal. The solution was then incubated for 4 hours at room temperature for proteoliposome formation.

4.6 Preparation of giant unilamellar vesicles from proteoliposomes

For the patch clamp electrophysiology experiments, we generated giant unilamellar vesicles (GUVs) containing Kv4.3 from proteoliposomes as prepared above. The GUVs were formed as previously described (Birkner, Poolman, & Kocer, 2012). Briefly, the proteoliposome solution was diluted in water to reach a final lipid concentration of 0.8 mg/mL and was spotted onto two ITO-covered glass slide to form an array of droplets (each droplet is 2 μ L). The droplets were dried in a vacuum chamber overnight. The giant unilamellar vesicles (GUVs) were obtained by electroformation using 300 mM sucrose solution and the application of pulsed voltage between the two ITO-covered glass slides using a Nanion Vesicle PrepPro instrument.

4.7 Acetone precipitation of protein from proteoliposome sample

One volume of proteoliposome solution was supplemented with 4 volumes of cooled (-20 °C) acetone. The solution was vortexed and incubated for 1 hour at -20 °C to precipitate proteins. The tube was centrifuged at 15.000 x g for 10'. The supernatant and the pellet were collected separately and stored at -20 °C.

4.8 Protein detection: Coomassie blue staining and Western blot

The protein samples were loaded on two 10% SDS-polyacrylamide gels. Once the proteins were separated by their molecular weight, the gel was either stained using Coomassie blue or used for Western blot. The proteins were first transferred from the gel onto a PVDF membrane; then the membrane was blotted using a primary antibody from mouse (1:1000, NeuroMab clone K75/41) and a secondary antibody against mouse (1:10.000, IR680 LI-COR). The gel and the membrane were imaged using an Odyssey® Fc imaging system (LI-COR).

4.9 Cell culture

CHO cells (Sigma) were seeded on sterile glass coverslips (~20,000 cells) in Dulbecco's Modified Eagle's Medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen) and 1% Penicillin-Streptomycin (Gibco, Rockville, MD). The plasmids pcDNA3.1-KCND3S (0.5 μ g) and the reporter pmaxGFP were transfected at a 1:1 molar ratio using Genius reagent (Westburg, Germany). Cells were incubated at 37 °C with 5% CO₂.

4.10 *Electrophysiology*

For the recording of channel activity in CHO cells, one coverslip was transferred to the recording chamber 24-48 hours after transfection. Only cells expressing the GFP were used for electrophysiological recording. Cell-attached and inside-out configurations were used for recording of single-channel activity. Channel activity was recorded at different membrane voltages (+40 mV, +50 mV, +60 mV, +70 mV and +80 mV) for 1' after the cell membrane was hyperpolarized at -90 mV for 30". The data was recorded using an Axopatch 200B amplifier, Digidata 1320 A interface and pClamp 8.2 software (Axon Instruments, Foster City, CA). The data was filtered at 5 kHz and sampled at 10 kHz. The same solution was used both in the bath and in the pipette (mM): 140 KCl, 10 ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid, 1 CaCl₂, 1 MgCl₂, 10 HEPES, pH 7.2. Microelectrodes were made from GC120F-10 borosilicate glass (Harvard Apparatus, Holliston, MA) and pulled on a P-87 puller (Sutter Instruments, Novato, CA) having a final resistance between 5 and 15 M Ω .

For recordings in giant liposomes, ~2 μ l liposome solution was diluted into 2 ml pipette solution (see paragraph above for composition). The liposomes were patched in the cell-attached mode using pipette with a resistance between 1 and 5 M Ω .

Data were analyzed using Clampfit (Axon Instruments, Foster City, CA). Low-pass digital filter was applied to all traces. If necessary, the baseline was manually adjusted.

5 References

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Chapter 7

Discussion and future perspectives

Claudio Tiecher

Ion channels are ubiquitous membrane proteins which allow the passage of ions across the cell membrane and are activated from different stimuli, such as voltage, ligand, and pressure (Hille, 1986). The voltage-gated potassium channel Kv4.3 forms a complex together with auxiliary and accessory proteins (*i.e.*, KChIPs, DPLPs and Kv β s), and it generates a fast-transient potassium current, known as somatodendritic A-type potassium current (I_{SA}) and transient outward potassium current (I_{to}) in the brain and the heart, respectively (Kong et al., 1998; Serôdio, Vega-Saenz de Miera, & Rudy, 1996). In neurons, this fast-transient current regulates neuronal firing frequency by dampening the incoming postsynaptic signal, whereas, in the heart, it is responsible for the repolarization of action potential (Nerbonne & Kass, 2005; Shibata et al., 2000). Cell-specific expression of regulatory proteins fine-tunes the I_{SA} and I_{to} , allowing the proper functioning of neurons and myocytes (Jerng, Pfaffinger, & Covarrubias, 2004; Patel, Campbell, Morales, & Strauss, 2002). In our brain, the Kv4 channel complex, hence the I_{SA} , plays an important role in the fine-tuning of motor and cognitive tasks. Specifically, the Kv4 channel complex is found in different type of cerebellar neurons, such as the granule (GrCs) and Purkinje cells (PCs), where it determines the electrical output of the cerebellum, and it ultimately modulates the activity of other brain regions which are involved in movement and cognition (D'Angelo, 2018).

Several mutations, which cause either ataxia or are linked to heart arrhythmias (*e.g.*, Brugada syndrome), have been identified in the loci encoding for the Kv4.3 channel subunit. Patients carrying ataxia-causing mutations suffer from motor and cognitive deficiencies. Several inherited and *de novo* mutations have been reported, and lead to the development of late and early onset cerebellar ataxia, respectively. Mutations which lead to late onset cerebellar atrophy are all loss-of-function and differentially affect the activity and the surface expression of the Kv4.3 channel complex (Duarri et al., 2012, 2013; Smets et al., 2015). Heart arrhythmias are a group of diseases which may lead to sudden cardiac arrest. Mutations, reported thus far, are gain-of-function mutations, and have been proposed to affect the repolarization phase of the action potential in the heart (Giudicessi et al., 2012).

First, in this thesis, we aimed at better understanding how inherited and *de novo* mutations affect the Kv4.3 channel function and structure (Chapter 2 and 3). We did this by applying different methodologies, including patch clamp, homology modelling, MD simulations, and immunocytochemistry. Second, we have established protocols in order to

investigate the Kv4.3 channel structure and function using biophysical methods (e.g., voltage clamp fluorometry and GUV patch clamp). Specifically, we have shown the feasibility to label the Kv4.3 channel using the unnatural amino acid ANAP (Chapter 4), and to functionally reconstitute the Kv4.3 channel into liposomes (Chapter 6). Third, in Chapter 5, we have used the information gained from studying patient mutations to better understand the molecular determinants of Kv4.3 channel conductance.

1 The role of mutations in the development of cerebellar pathophysiology

1.1 *Mutations affect macroscopic as well as microscopic properties of Kv4.3*

We have shown that mutations in *KCND3* encoding the Kv4.3 channel do not only affect macroscopic aspects of channel functioning (e.g., current density, steady-state, and kinetics). Specifically, in Chapter 2, 3, and 5, we have shown how missense mutations affect the electrophysiological properties at the microscopic level (*i.e.*, unitary conductance). Different studies have also reported the effect of point mutations on the unitary conductance of BK channels and Shaker channel (Brelidze & Magleby, 2005; Moscoso et al., 2012).

These observations stress the importance to study the effect of mutations linked to disease on macroscopic (e.g., channel stability, trafficking, and modulation by auxiliary proteins) as well as microscopic properties (e.g., unitary conductance and dwell time). As shown in Chapter 2, the SCA19/22 mutations cause a decrease in the single-channel currents. This decrease partly contributes to the overall drop in potassium whole-cell currents generated by the mutated channels. Our work confirms the idea that unitary conductance is not a fixed and immutable properties, on the contrary, is a dynamic and changing one (Naranjo, Moldenhauer, Pincuntureo, & Díaz-Franulic, 2016). Based on these observations, we advise future studies to include the study of microscopic properties in order to fully describe the pathophysiology of channelopathies.

1.2 *The importance to validate genetic variants using functional assays*

In Chapter 3, we have investigated the effect of *de novo* mutations on the Kv4.3 channel function, and we have found that the majority of *de novo* mutations cause a severe disease with an early age of onset compared to inherited mutations. In the genome of the average individual, we find

44 to 82 *de novo* mutations. Only one or two of these *de novo* mutations are found in the coding sequence and in most case do not cause any disease. Damaging *de novo* mutations arise in the germline, do not undergo purifying selection, and cause diseases (e.g., Schinzel-Giedion syndrome, Kabuki syndrome) with a severe early onset phenotype (Hoischen et al., 2010; Ng et al., 2010). Inherited pathogenic mutations, instead, survive evolutionary selection, can be passed on to the offspring, and usually lead to the development of mild diseases with a late onset (Acuna-Hidalgo, Veltman, & Hoischen, 2016; Veltman & Brunner, 2012). Given the severity of the clinical phenotype, we expected that *de novo* mutations have also a severe effect on channel functioning, as was shown for the *de novo* RVF duplication in *KCND3* (Smets et al., 2015).

As expected, the majority of *de novo* mutations (i.e., V294F, L310P, T366I, and G371R), studied in Chapter 3, alter channel conductance at physiologically relevant potentials via different mechanisms. The V294F mutation shifts the A-type current to more depolarized potentials, the L310P and T366I mutant channels do not localize at the plasma membrane resulting a complete absence of A-type current, and the G371R mutant channel does not generate any current due to blockage of the channel pore. However, the V399L mutation has only mild effects on the functioning of the Kv4.3 channel, even milder than the effect of the S390N inherited mutation, located within the same protein domain, and does also not affect trafficking and PM localization of Kv4.3. Thus, based on our functional data we cannot classify this mutation as pathogenic and may be benign.

We may speculate that the pathogenic effect of the V399L mutation emerges via its interaction with auxiliary subunits (see discussion of Chapter 3) or has an effect on neuronal firing as in the case of SCA42, where the R1723H mutation in Ca_v3.1 with mild effect on channel activity does significantly affect neuronal firing frequency (Hashiguchi et al., 2019). Our work on the V399L mutation stresses the importance to handle the information obtained from human genetic studies with care before a conclusion can be made on pathogenicity. Particularly, when mutations are found in one isolated individual, the causal effect of mutations has to be established on the basis of, not only genetic, but also functional approaches (e.g., electrophysiology) (Hampel, Bennett, Buchanan, Pearlman, & Wiesner, 2015; Smith et al., 2017; Veltman & Brunner, 2012).

1.3 *The effect of loss-of-function mutations in the Kv4.3 channel on neuronal excitability of Purkinje cells*

Channelopathies including SCA19/22 (e.g., episodic ataxia type 2, SCA6, SCA13, SCA15/16/29, SCA27, SCA41, and SCA42) which result in cerebellar dysfunction are caused by genetic variations in genes encoding calcium, potassium, and sodium channels (Bushart & Shakkottai, 2018; Klockgether, Mariotti, & Paulson, 2019). Several of these malfunctioning mutant ion channels lead to the development of ataxia as a result of improper altered firing activity of PCs, in some cases complicated by a neuronal development component (Becker et al., 2009; Hashiguchi et al., 2019; Irie, Matsuzaki, Sekino, & Hirai, 2014; Issa, Mazzochi, Mock, & Papazian, 2011). For instance, in the case of mutations in *CACNA1A* encoding the voltage-gated calcium channel $\text{Ca}_v2.1$, gain- and loss-of-function mutations cause cerebellar ataxia by disrupting the firing pattern of PCs (Fletcher et al., 1996; Gao et al., 2012). Since both an increase and a reduction of calcium influx cause malfunctioning of PCs, another study suggested that a narrow window of Ca^{2+} concentrations needs to be maintained in order to ensure the proper functioning of PCs (De Zeeuw et al., 2011). In the case of $\text{Ca}_v3.1$ channel, the R1723H mutation causes a modest shift in the voltage activation of the channel which is sufficient to alter the firing of PCs and lead to the development of SCA42 (Hashiguchi et al., 2019).

We have shown in Chapter 2 and 3, that inherited and *de novo* mutations cause a reduction in the availability of I_{SA} . This results from a reduced number of Kv4.3 channels localizing at the PM as well as a reduction or complete loss of Kv4.3 channel conductance at subthreshold membrane voltages. In humans, after a hyperpolarization event, the I_{SA} briefly activates and inactivates, when the cell starts depolarizing and the membrane potential has not yet reached its firing threshold (~ -50 mV). The presence of this current results in the suppression of membrane depolarization events in neuronal cells. Whether a given depolarization event is suppressed, it depends on the intrinsic kinetics properties of the Kv4 channel complex, which ultimately determines the timing of action potential firing (Shibata et al., 2000). Thus our work may suggest that the lack of I_{SA} would likely translate into decreased firing latency and an overall increase in neuronal excitability in PCs expressing the mutated Kv4.3 channel in the presence of auxiliary proteins (Figure 1A-B).

Neuronal hyperexcitability has already been associated with a number of diseases affecting the brain, such as Alzheimer's disease, epilepsy, Parkinson's disease, and tinnitus (Chopra, Bushart, & Shakkottai, 2018).

Figure 1 The proposed effect for the lack of the I_{SA} on action potential, neuronal firing, and mitophagy in neurons. (A) Two single action potentials are depicted. The slow one takes longer time to reach the firing threshold as a result of suppressed depolarization events compared to the fast one. (B) Hypothetical depiction of neuronal firing. Neurons which lack the I_{SA} show a higher activity compared to the ones harboring the $Kv4$ channel complex. (C) Proposed mechanism leading to excitatory mitochondrial toxicity (EMT) upon sustained depolarization events. In the presence of $Kv4$ channels (left), depolarization events are dampened from spreading along the cell membrane. As a result, voltage-gated calcium channels do not repeatedly and intracellular calcium concentration is maintained within physiological levels. In the absence of $Kv4$ channels, voltage-gated calcium channels are constantly active resulting in high level of calcium in the cytosol. This drives up calcium uptake from mitochondria which eventually undergo mitophagy and cause dendrite retraction. Sodium, $Kv4$, voltage-gated calcium channels, and corresponding ions are depicted in green, orange/red, and blue, respectively. The presence and the absence of I_{SA} is shown in black and red.

In the case of cerebellar ataxia, hyperexcitability may cause excitatory mitochondrial toxicity (EMT) which causes an increase in mitochondria degradation, eventually leading to dendrite retraction and cell death. EMT would likely take place around calcium entry sites (i.e., dendrites) in

neurons with a high metabolic rate (e.g., Purkinje cells). PCs are the most metabolically and electrically active neurons in the whole brain, and are thus a very likely target of EMT (Figure 1C). We hypothesize that calcium may accumulate inside the cytosol and mitochondria as a result of increased activation of voltage-gated calcium channels due to the absence or reduction of I_{SA} . High levels of intracellular calcium would trigger EMT and eventually lead to PCs' death (Chen, 2005; Hall et al., 2015; Morse, 2010; Verma, Wills, & Chu, 2018). Future studies are needed to investigate this hypothesis and examine the effect of mutations in the Kv4.3 channel on firing frequency, calcium levels, and mitochondria degradation in cerebellar neurons including PCs.

1.4 *Mutations in KCND3 have an effect on the modulation of Kv4.3 channel activity by its auxiliary proteins*

The Kv4.3 channel constitutes the conducting portion of the Kv4 channel complex which, *in vivo*, also includes auxiliary proteins (i.e., KChIP and DPLP). As a whole, the channel complex generates the A-type potassium current. In Chapter 2 and 3, we showed that inherited and *de novo* mutations alter the way the auxiliary subunit KChIP2b modulates the Kv4.3 channel. This suggests that mutations in the *KCND3* coding sequence have an indirect effect on their modulation by the auxiliary subunits. Future experiments will need to investigate the effect of mutations, not only on Kv4.3 channel function, but also on its interaction and modulation by the auxiliary proteins.

2 What inherited and *de novo* mutations taught us about the working mechanism of the Kv4 channel complex

2.1 *The interaction between KChIP2b and the Kv4.3 channel*

Our functional studies have shown that inherited and *de novo* mutations affect the modulatory effects of the auxiliary subunit KChIP2b on Kv4.3 (Chapter 2 and 3). Our findings suggest that structural alterations due to mutations in Kv4.3 channel hamper the activity of KChIP2b. These mutations are located within the voltage-sensing domain (VSD) and the pore domain (PD), both not directly interacting with KChIP2b based on currently known interaction interfaces (Pioletti, Findeisen, Hura, & Minor, 2006; Wang et al., 2007). We hypothesize that KChIP2b, might have other interaction sites on the Kv4.3, that has not been yet identified, as also suggested by others (Kim et al., 2004; Pioletti et al., 2006).

Structural and functional studies have established that KChIP binds to the N-terminal cytosolic domain of the Kv4 channel and modulate channel activity (Pioletti et al., 2006; Wang et al., 2007). Although it was initially

thought that KChIP exerts its function by binding the N-terminus of Kv4, KChIP modulates the activity of the Kv4 channel even in the absence of the N-terminus (Bähring et al., 2001; Barghaan, Tozakidou, Ehmke, & Bähring, 2008; Jerng & Covarrubias, 1997). This suggests that KChIP modulates channel activity by interacting with the VSD and the PD via the T1 domain (Pioletti et al., 2006; Wang et al., 2007). This KChIP-T1-VSD/PD long range interaction may explain why mutations in the transmembrane domains (TMDs) of the Kv4.3 channel cause changes in the activity of KChIP2b. However, how these three domains (*i.e.*, KChIP, T1, and VSD/PD) are mechanistically coupled remains unclear. Future structure-function studies will have to elucidate and confirm how KChIP modulates the activity of the Kv4.3 channel.

2.2 The role for the methionine residue at the exit of the channel pore
In potassium channels, the pore domain contains a highly conserved sequence, namely the selectivity filter (SF). This acts as a molecular sifter which is thousands of times more permeable to potassium than sodium. Moreover, the SF determines unitary conductance, as shown from studies on the Shaker and Kv2.1 channels (MacKinnon & Yellen, 1990; Trapani, Andalib, Consiglio, & Korn, 2006).

In the case of the Kv4.3 channel, we have shown that the SF also plays an important role in determining unitary conductance (Chapter 2, 3, and 5). In addition, we have found that the Kv4.3 channel with an unnatural M373E variation activates at more hyperpolarized membrane potentials compared to WT (Chapter 5). Our work has highlighted the role of the residue at position 373 in tweaking channel function. This knowledge may be used to engineer channel properties *in vivo* or *in silico* in order to correct malfunctioning of ion channels.

3 Tools for the future

Many questions remain unanswered regarding the effect of mutations on the structure and function of the Kv4.3 channel complex. How KChIP2b modulates the activity of the Kv4.3 channel possibly by acting on the T1 domain is not yet understood. Moreover, how the M373E mutation causes the channel to generate currents at more hyperpolarized potentials compared to WT remains unclear. Last, how different membrane lipids modulate the function of the Kv4.3 channel is still an open question. These research questions cannot be addressed using *in silico* approaches (*i.e.*, MD simulations), since computational studies are currently limited to short time scales (< 1 ms) which cannot cover structural changes involved in the activation and inactivation of ion channels (DeMarco, Bekker, &

Vorobyov, 2019). The limitations of MD can be overcome by using biophysical methods, such as voltage clamp fluorometry and patch clamp of giant unilamellar vesicles (Chapter 4 and 6).

In Chapter 4, we labeled the Kv4.3 channel with a fluorescent probe (*i.e.*, ANAP). This will allow us to use biophysical techniques (*e.g.*, spectrometry and fluorometry) to simultaneously follow structural and functional changes in the Kv4.3 channel. In Chapter 6, we have presented an *in vitro* platform which allows researchers to study the effect of various cellular components (*i.e.*, lipids, phosphorylation agents, peptides), which cannot be tested using conventional patch clamp methods, on channel function. Moreover, these protocols may be combined to allow structure-function studies in a defined biological environment.

4 General conclusion and outlook

In this thesis, we have characterized the effect of several mutations on Kv4.3 channel function in the presence and absence of KChIP2b. These mutations cause all a loss-of-function of the channel and result in the lack of inhibitory potassium current in cells and in MD simulations. We hypothesize that the lack of or reduced potassium currents may lead to an increase in the activity of voltage-gated calcium channels, ultimately leading to toxic levels of intracellular calcium and subsequent neuronal dysfunction and/or cell death.

Each of these mutations caused a reduction in potassium conductance by differentially affecting the structure and the function of the WT Kv4.3 channel complex. This heterogeneity may help us in part explain the clinical diversity of patients' phenotypes, but will pose a major challenge for the development of a common treatment.

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Addendum

Summary - List of abbreviations - Acknowledgments

1 Summary

Neuronal cells are the basic building blocks of the nervous system and allow us to perform motor and cognitive tasks. The main function of these cells is to receive and relay information along the neuronal network. Information travels from one neuron to the next as an electrical stimulus, known as action potential. Action potentials are generated from the concerted activity of ion channels, specialized membrane proteins which allow the passage of ions across the cell membrane. Voltage-gate ion channels activate upon changes in the membrane potential. Specifically, the voltage-gated potassium channel Kv4.3 activates at subthreshold potential and generates a fast-transient so-called A-type outward current. In the brain, the A-type current dampens incoming electrical stimuli and blocks backpropagation of action potential, ultimately modulating neuronal firing frequency.

The Kv4.3 channel possesses a voltage-sensing domain (VSD). The VSD is responsible for sensing voltage changes within the cell membrane, which results in structural rearrangements that open the pore domain (PD) of the channel. Once open, the PD allows the passage of potassium ions, thus generating an outward potassium current. Two auxiliary proteins (Kv Channel-Interacting Proteins and Dipeptidyl Peptidase-Like Proteins) bind to the Kv4.3 channel and modulate its function. They increase the surface expression of the Kv4.3 channel, with the exception of KChIP4a, and affect the kinetics and voltage of activation and inactivation of the Kv4.3 channel.

Malfunctioning ion channels cause ion channels disorders, known as channelopathies. In the case of Kv4.3, inheritable and *de novo* mutations result in the development of spinocerebellar ataxia 19/22 (SCA19/22) and a complex clinical phenotype, respectively. The latter includes not only ataxia but also extracerebellar symptoms, such as intellectual disability and myoclonus. Ataxic patients who carry inheritable or *de novo* mutations have usually problems keeping a steady gait. Patients who suffer from extracerebellar symptoms can have difficulties in school and are unable to coordinate the movement of head, arms, and feet. Although previous studies have studied the effect of these mutations on the surface expression and stability of the Kv4.3 protein, the disease mechanism is not fully understood.

In this thesis, we studied the impact of patient-derived and *in silico* mutations on the structure and function of the Kv4.3 channel. First, we characterized the effect of several reported SCA19/22 and novel *de novo*

mutations on the activity of the Kv4.3 channel complex (Kv4.3-KChIP2b) in a cell model. Next, by employing molecular dynamics simulations, we could identify the underlying structural changes for the observed functional alterations (Chapter 2 and 3). Second, we studied how a SCA19/22 mutation (M373I) located within the channel pore modulates the Kv4.3 channel properties by comparing its functional consequences with an *in silico* predicted mutation on the same amino acid using structural modelling (Chapter 5). Moreover, we developed biophysical tools (*i.e.*, voltage clamp fluorometry using genetically encoded unnatural amino acid and liposome reconstituted with functional Kv4.3 channels) to allow future investigations of the Kv4.3 channel structure and function (Chapter 4 and 6). These studies were conducted using different techniques, namely electrophysiology (*i.e.*, patch clamp and two electrode voltage clamp) and molecular modelling. The first one allowed us to study the effect of mutations on the ability of the Kv4.3 channel to generate electrical currents. The second one provided us with detailed information about the effect of mutations on the structure of the Kv4.3 channel. Especially, molecular modelling was the only tool available to examine the activity of mutant channels (*i.e.*, G371R) that localize at the plasma membrane, but do not generate any current.

The main findings of this thesis can be summarized as follow:

- inherited and *de novo* mutations in *KCND3* reduce the amount of current generated by the Kv4.3 channel by differentially affecting its structure and function.
- inherited and *de novo* mutations in *KCND3* affect the modulatory effect of the auxiliary subunit KChIP2b on the Kv4.3 channel activity.
- the hydration state of the Kv4.3 channel pore affects single-channel conductance
- the fluorescent amino acid ANAP can be used to label the Kv4.3 channel and, in the future, simultaneously monitor structural and functional changes in the Kv4.3 channel
- the Kv4.3 channel can be reconstituted into brain lipid extract and this platform will allow the investigation of the Kv4.3 channel complex from the bottom up

These results strengthen the idea that disease-causing mutations result in the reduction of currents generated by the Kv4.3 channel in neurological conditions. Moreover, our work suggests that mutations do not only affect the Kv4.3 channel activity, but also the activity of modulatory subunits (*e.g.*, KChIP2b). In the future, researchers will have to study the effect of mutations on the Kv4.3 channel in combination with auxiliary subunits expressed in affected neuronal cells types (*e.g.*, Purkinje cells).

Molecular modelling has proven to be an essential tool to shed light on the structural changes generated by mutations. This tool should be

included in future investigations to fully describe the effect of mutations on the channel function and structure.

The biophysical tools developed in this thesis showed that studying the Kv4.3 channel complex from the bottom up is experimentally feasible. In the future, this approach will allow us to decipher the functional heterogeneity of the Kv4.3 channel complex by examining the role of individual components (e.g., lipids and auxiliary proteins) on the Kv4.3 channel activity.

2 Samenvatting

Zenuwcellen zijn de bouwstenen van het zenuwstelsel, ze spelen een essentiële rol bij de uitvoering van alle motorische en cognitieve taken. De belangrijkste functie van zenuwcellen is het ontvangen en versturen van informatie over het neuronale netwerk. De informatie wordt van het ene naar het andere neuron doorgegeven door middel van een elektrische stimulus, het actiepotentiaal. Actiepotentialen worden gegenereerd door de gecoördineerde activiteit van ionkanalen, dit zijn gespecialiseerde membraan eiwitten die zorgen voor het passieve transport van ionen door het celmembraan. Spanninggeactiveerde ionkanalen worden geactiveerd wanneer er een verandering optreedt in het membraanpotentiaal. Het spanning geactiveerde kaliumkanaal Kv4.3 wordt geactiveerd wanneer het membraanpotentiaal onder de grenswaarde daalt. Activatie van het Kv4.3 kanaal genereert een snelle kortdurende uitgaande stroom, de zogenaamde A-type stroom. In de hersenen leidt dit tot demping van inkomende elektrische signalen en het blokkeren van terugwaartse geleiding van actiepotentialen, met als gevolg een verandering in de vuurfrequentie van het neuron.

Het Kv4.3 kanaal bezit een spanningsdetectie domein (SDD). Het SDD is verantwoordelijk voor het detecteren van spanningsveranderingen in het celmembraan en vervolgens voor opening van het porieendomein (PD) door structurele herschikking. Eenmaal geopend, maakt het PD de doorgang van kaliumionen mogelijk, waardoor een uitgaande kaliumstroom ontstaat. Twee accessoire eiwitten kunnen de functie van het Kv4.3 kanaal moduleren (Kv Channel-Interacting Proteins en Dipeptidyl Peptidase-Like Proteins). Door aan het Kv4.3-kanaal te binden verhogen ze de expressie van het kanaal in het plasmamembraan, beïnvloeden ze de kinetiek en de spanning voor het activeren en deactiveren van het Kv4.3 kanaal, met uitzondering van KChIP4a.

Wanneer een ionkanaal defect is, kan dit leiden tot een ziekte van het ionkanaal (kanalopathie). In het geval van Kv4.3 leiden erfelijke mutaties tot ontwikkeling van spinocerebellaire ataxie 19/22 (SCA19/22) en *de novo* mutaties tot een complex klinisch fenotype. Het complexe fenotype omvat niet alleen ataxie maar ook extra-cerebellaire symptomen, zoals verstandelijke beperkingen en myoklonieën. Patienten met ataxie ten gevolge van erfelijke of *de novo*-mutaties hebben meestal moeite om het evenwicht te bewaren en stabiel te kunnen lopen. De extra-cerebellaire symptomen kunnen zich uiten in: problemen op school en het niet kunnen coördineren van de bewegingen van hoofd, armen, en voeten. Hoewel eerdere studies het effect van deze mutaties op de oppervlakte expressie

van het Kv4.3 eiwit in het celmembraan hebben bestudeerd, is de pathogenese niet volledig bekend.

In dit proefschrift hebben we het effect bestudeerd van bestaande en *in silico* mutaties op de structuur en de functie van het Kv4.3 kanaal. Ten eerste hebben we het effect van verschillende bekende SCA19/22 en nieuwe *de novo* mutaties op de activiteit van het Kv4.3 kanaalcomplex (Kv4.3KChIP2b) in een celmodel gekarakteriseerd. Vervolgens konden we met behulp van moleculaire dynamica simulaties de onderliggende structurele veranderingen identificeren voor de waargenomen functionele veranderingen (Hoofdstuk 2 en 3). Ten tweede hebben we onderzocht hoe een SCA19/22 mutatie (M373I), die zich in de kanaalporie bevindt, de eigenschappen van het Kv4.3 kanaal moduleert, door de functionele gevolgen te vergelijken met een *in silico* voorspelde mutatie van hetzelfde aminozuur met behulp van structurele modellering (Hoofdstuk 5). Bovendien hebben we biofysische hulpmiddelen ontwikkeld (d.w.z., spanningsklem fluorometrie, met behulp van genetisch gecodeerd onnatuurlijk aminozuur en liposoom gereconstitueerd met functioneel Kv4.3 eiwit) om toekomstig onderzoek naar de structuur en functie van het Kv4.3 kanaal mogelijk te maken (Hoofdstuk 4 en 6). Deze studies werden uitgevoerd met behulp van verschillende technieken, namelijk elektrofysiologie (d.w.z., patch clamp en two electrode voltage clamps) en moleculaire modellering. De eerste techniek stelde ons in staat om het effect van mutaties op het vermogen van Kv4.3 om elektrische stromen te genereren te bestuderen. De tweede techniek gaf ons gedetailleerde informatie over het effect van mutaties op de structuur van het Kv4.3 kanaal. Moleculaire modellering was het enige beschikbare hulpmiddel om de activiteit van de gemuteerde kanalen (d.w.z., G371R) te onderzoeken die zich in het plasmamembraan bevinden maar geen stroom genereren.

- de belangrijkste bevindingen van dit proefschrift kunnen als volgt worden samengevat
- erfelijke en *de novo* mutaties in *KCND3* verminderen de hoeveelheid stroom die wordt gegenereerd door het Kv4.3 kanaal door verschillende veranderingen in de structuur en de functie van het kanaal
- erfelijke en *de novo* mutaties in *KCND3* beïnvloeden het modulerende effect van de hulpsubeenheid KChIP2b op de activiteit van het Kv4.3 kanaal
- de hydratatietoestand van porieendomein beïnvloedt de single-channel conductance van het Kv4.3 kanaal
- het fluorescerende aminozuur ANAP kan worden gebruikt om het Kv4.3-kanaal te labelen en in de toekomst tegelijkertijd structurele en functionele veranderingen in het Kv4.3-kanaal te monitoren

- het Kv4.3-kanaal kan worden gereconstitueerd in hersenlipide extract en door middel van dit platform kan het Kv4.3 complex bottom-up worden onderzocht

Deze resultaten versterken het idee dat ziekteverwekkende mutaties resulteren in vermindering van elektrische stromen die worden gegenereerd door het Kv4.3 kanaal in zenuwaandoeningen. Bovendien suggereert ons werk dat mutaties niet alleen de activiteit van het Kv4.3 kanaal beïnvloeden, maar ook de activiteit van accessoire eiwitten (*bijv.*, KChIP2b). In de toekomst zullen onderzoekers het effect van mutaties op het Kv4.3 kanaal moeten bestuderen in combinatie met hulp-subeenheden die tot expressie worden gebracht in aangetaste neuronale celtypen (*bijv.*, Purkinje cellen).

Moleculaire modellering is een essentieel hulpmiddel gebleken om de structurele veranderingen die door mutaties worden veroorzaakt te onderzoeken. Dit hulpmiddel moet worden opgenomen in toekomstige onderzoeken om het effect van mutaties op de kanaalfunctie en structuur volledig te kunnen begrijpen.

De biofysische hulpmiddelen die in dit proefschrift zijn ontwikkeld, toonden aan dat het Kv4.3 complex bottom-up bestudeerd kan worden. In de toekomst zal deze benadering het mogelijk maken om de functionele heterogeniteit van het Kv4.3 eiwitcomplex te ontcijferen door de rol van individuele componenten (*bijv.*, lipiden en accessoire eiwitten) op de activiteit van het Kv4.3 kanaal te onderzoeken.

3 Riassunto

Le cellule neuronali costituiscono le fondamenta del nostro sistema nervoso e ci consentono di muoverci e pensare. La funzione principale di queste cellule è quella di ricevere e trasmettere informazioni lungo la rete neuronale. Le informazioni viaggiano da un neurone all'altro sotto forma di stimoli elettrici, chiamati anche potenziali d'azione. Questi sono generati dall'attività di diversi canali ionici, proteine di membrana specializzate nel facilitare il passaggio di ioni attraverso la membrana cellulare. Alcuni canali ionici, noti come Kv, sono voltaggio-dipendenti e si attivano quando il potenziale di membrana cambia. In particolare, il canale ionico conosciuto come Kv4.3 si attiva a potenziali di membrana al di sotto del "valore soglia", necessario per la generazione del potenziale d'azione. Quando attivo, il Kv4.3 genera una corrente di ioni potassio, nota come tipo A, che viaggia dall'interno all'esterno della membrana cellulare. Nel cervello, la corrente di tipo A smorza gli stimoli elettrici in entrata e blocca la propagazione in senso retrogrado del potenziale d'azione. Questa corrente contribuisce a modulare la frequenza con la quale le cellule neuronali rispondono a stimoli elettrici esterni.

Il Kv4.3 possiede un dominio proteico (VSD) il quale è responsabile per il rilevamento di variazioni nel potenziale di membrana risultando in cambiamenti strutturali che portano all'apertura del poro (PD) del canale. Una volta aperto, il PD consente il passaggio di ioni potassio, generando così una corrente di potassio verso l'esterno della cellula. Due proteine modulatrici (note come Kv Channel-Interacting Proteins and Dipeptidyl Peptidase-Like Proteins) interagiscono con il Kv4.3 e ne regolano la sua funzione. Queste proteine modulatrici aumentano l'espressione di Kv4.3 all'interno della membrana cellulare, con l'eccezione di KChIP4a, e influenzano la cinetica e i valori di voltaggio che portano all'attivazione/inattivazione del Kv4.3.

Nell'uomo, disfunzioni dei canali ionici portano allo sviluppo di diverse malattie, note come canalopatie. Nel caso del Kv4.3, mutazioni ereditarie e *de novo* provocano rispettivamente lo sviluppo dell'atassia spinocerebellare 19/22 (SCA19/22) e altri profili clinici complessi. Questi ultimi includono non solo atassia ma anche altri sintomi come disabilità intellettiva e mioclono. Pazienti che presentano mutazioni ereditarie o *de novo* hanno generalmente problemi a mantenere un'andatura stabile e, in alcuni casi, hanno difficoltà cognitive e non sono in grado di coordinare il movimento di testa e arti. Sebbene precedenti ricerche scientifiche abbiano studiato l'effetto di queste mutazioni sull'espressione all'interno

della membrana cellulare e sulla stabilità della proteina Kv4.3, il meccanismo che porta alla malattia non è completamente compreso.

In questa tesi, abbiamo studiato l'impatto di mutazioni riscontrate in pazienti e previste *in silico* sulla struttura e funzione del Kv4.3. Innanzitutto, abbiamo caratterizzato l'effetto di diverse mutazioni ereditarie e *de novo* sull'attività del complesso formato dal Kv4.3 con KChIP2b in un modello cellulare. Successivamente, impiegando simulazioni di dinamica molecolare, abbiamo identificato i cambiamenti strutturali alla base delle alterazioni funzionali osservate (Capitolo 2 e 3). In secondo luogo, abbiamo studiato come una mutazione (M373I), causante SCA19/22, situata all'interno del poro influenza le proprietà di Kv4.3. Questo è stato possibile confrontando gli effetti di tale mutazione con una mutazione prevista *in silico* (Capitolo 5). Inoltre, abbiamo sviluppato strumenti biofisici (*i.e.*, fluorometria associata con voltage clamp usando amino acidi sintetici e liposomi ricostituiti con il Kv4.3) per consentire future ricerche sulla struttura e sulla funzione del Kv4.3 (Capitolo 4 e 6). Questi studi sono stati condotti utilizzando diverse tecniche, come elettrofisiologia (*i.e.*, patch clamp e voltage clamp con due elettrodi) e modellistica molecolare. Il primo ci ha permesso di studiare l'effetto delle mutazioni sulla possibilità per Kv4.3 di generare correnti elettriche. Il secondo ci ha fornito informazioni dettagliate sull'effetto delle mutazioni sulla struttura del Kv4.3. In particolare, la modellistica molecolare era l'unico strumento disponibile per esaminare l'attività di canali mutanti (*i.e.*, G371R) che sono inseriti nella membrana cellulare, ma non generano corrente.

I principali risultati di questa tesi possono essere riassunti come segue:

- mutazioni ereditarie e *de novo* nel gene *KCND3* riducono la quantità di corrente generata dal canale Kv4.3 la cui struttura e funzione sono differenzialmente alterate
- mutazioni ereditarie e *de novo* nel gene *KCND3* influenzano variazioni indotte da parte della proteina modulatrice KChIP2b sull'attività del Kv4.3
- lo stato di idratazione del poro nel Kv4.3 influenza la conduttanza del canale a livello microscopico
- l'amminoacido fluorescente ANAP può essere usato per marcare il Kv4.3 e, in futuro, monitorare simultaneamente i cambiamenti strutturali e funzionali in tale canale
- il Kv4.3 può essere ricostituito in estratto lipidico cerebrale e questo metodo consentirà di investigare il complesso del canale Kv4.3 partendo dal basso e andando verso l'alto (bottom up)

Questi risultati rafforzano l'idea che mutazioni causanti malattie neurologiche comportano la riduzione di correnti generate dal Kv4.3.

Inoltre, il nostro lavoro suggerisce che le mutazioni non influenzano solo l'attività del Kv4.3, ma anche l'attività delle proteine modulatrici (*i.e.*, KChIP2b). In futuro, i ricercatori dovranno studiare l'effetto delle mutazioni sul Kv4.3 in combinazione con proteine modulatrici che sono normalmente espresse in neuroni del cervelletto (*i.e.*, cellule del Purkinje).

Abbiamo dimostrato che la modellistica molecolare è uno strumento essenziale per far luce sui cambiamenti strutturali generati da mutazioni. Questo strumento dovrebbe essere incluso in future indagini per descrivere appieno l'effetto delle mutazioni sulla funzione e sulla struttura del canale.

Gli strumenti biofisici sviluppati in questa tesi hanno dimostrato che lo studio del Kv4.3 partendo dal basso e andando verso l'alto (bottom up) è sperimentalmente possibile. In futuro, questo approccio consentirà di decifrare l'eterogeneità funzionale di complessi formati dal Kv4.3 esaminando l'effetto dei singoli componenti (*i.e.*, lipidi e proteine modulatrici) sull'attività del Kv4.3.

4 List of abbreviations

AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANAP	3-(6-acetylnaphthalen-2-ylamino)-2-aminopropanoic acid
azF	p-azido-L-phenylalanine
BrS	Brugada syndrome
BzF	p-benzoyl-L-phenylalanine
CaCl ₂	calcium chloride
CHO	chinese hamster ovary
CiVSP	voltage-sensing phosphatase
CF	climbing fiber
CO ₂	carbon dioxide
cRNA	coding ribonucleic acid
cryo-EM	cryogenic electron microscopy
CPK	Corey-Pauling-Koltun
CSI	closed-state inactivation
DCN	deep cerebellar nuclei
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DPLP	dipeptidyl peptidase-like proteins
EGTA	ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid
EM	electron microscopy
EMT	excitatory mitochondrial toxicity
EPSP	excitatory post synaptic potentials
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
ESI	electrospray ionization
GABA	gamma-aminobutyric acid
GoC	Golgi cell
GrC	granule cell
GUV	giant unilamellar vesicle
HCl	hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hSERT	human serotonin transporter
IO	inferior olive
IPSP	inhibitory postsynaptic potentials
I _{SA}	somatodendritic subthreshold A-type potassium current
I _{to}	transient outward potassium current
ITO	indium-tin-oxide
K ⁺	potassium ion
KCl	potassium chloride
KCND1/2/3	gene name encoding for the potassium voltage-gated channel1/2/3
KChIP	Kv channel-interacting proteins

KNO ₃	potassium nitrate
KOH	potassium hydroxide
Kv	voltage-gate potassium channels
Kvβ	voltage-gated potassium channel subunit beta
MD	molecular dynamics
MF	mossy fiber
MgCl ₂	magnesium chloride
MLI	molecular layer interneurons
MM	molecular modelling
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MS	mass spectroscopy
NaCl	sodium chloride
NaOH	sodium hydroxide
NaNO ₃	sodium nitrate
Ni-NTA	nickel-nitrilotriacetic acid
NMR	nuclear magnetic resonance
LC	liquid chromatography
OD	optic density
OSI	open-state inactivation
PC	Purkinje cell
PDB	protein data bank
PCR	polymerase chain reaction
PD	pore domain
PIP ₂	phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PM	plasma membrane
PMSF	phenylmethylsulfonyl fluoride
POPC	phosphatidylcholine
PUFA	polyunsaturated fatty acids
PVDF	polyvinylidene difluoride
RMSD	root-mean-square deviations
RNA	ribonucleic acid
RT-PCR	real time polymerase chain reaction
SASA	solvent accessible surface area
SCA	spinocerebellar ataxia
SCD	sudden cardiac death
SCN1A	gene encoding for the sodium voltage-gated channel 1
SD	
SDS-PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SF	selectivity filter
T4L	T4 ligase

TEA	tetraethylammonium
TEVC	two-electrode voltage clamp
tRNA	transfer ribonucleic acid
TMD	transmembrane domain
VCF	voltage-clamp fluorometry
V_{rest}	resting membrane potential
VSD	voltage-sensing domain
WT	wild-type

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