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Abstract

Voltage-gated ion channels are crucial for both neuronal and cardiac excitability. Decades of research have begun to unravel the intriguing machinery behind voltage sensitivity. Although the details regarding the arrangement and movement in the voltage-sensing domain are still debated, consensus is slowly emerging. There are three competing conceptual models: the helical-screw, the transporter, and the paddle model. In this review we explore the structure of the activated voltage-sensing domain based on the recent X-ray structure of a chimera between Kv1.2 and Kv2.1. We also present a model for the closed state. From this we conclude that upon depolarization the voltage sensor S4 moves ~13 Å outwards and rotates ~180º, thus consistent with the helical-screw model. S4 also moves relative to S3b which is not consistent with the paddle model. One interesting feature of the voltage sensor is that it partially faces the lipid bilayer and therefore can interact both with the membrane itself and with physiological and pharmacological molecules reaching the channel from the membrane. This type of channel modulation is discussed together with other mechanisms for how voltage-sensitivity is modified. Small effects on voltage-sensitivity can have profound effects on excitability. Therefore, medical drugs designed to alter the voltage dependence offer an interesting way to regulate excitability.

Introduction

Nervous impulses are transmitted along axons at velocities up to 120 m/s. To perform this work, the cell membrane quickly and accurately changes its permeability to various ions [1]. This permeability is mediated by selective ion channels forming transmembrane pores. The channels are opened and closed by different stimuli such as neurotransmitters, membrane stretch, temperature, and transmembrane voltage. Depending on the design of the pore, they are selective for specific ions. The permeability changes necessary for transmitting the nervous impulse are caused by the opening and closing of different voltage-gated ion channels that respond to changes in the membrane potential [2]. Dysfunctional channels cause disease [3] and a large number of medical drugs, as well as animal and plant toxins, target ion channels. In general, most medical drugs in clinical use targeting ion channels block the ion-conducting pore. However, the voltage-sensing machinery is an alternative and suitable target for medical drugs that could tune channel activity and thereby also neuronal and cardiac excitability. While the ion-conducting pore is known at atomic resolution for several ion channels [4-8], the mechanism by which the channels sense transmembrane voltage is known in less detail [e.g. 9, 10]. In the present review, we focus on recent developments in understanding the voltage-sensing mechanism of voltage-gated ion channels, and on recent attempts to link medical drugs and other substances in targeting the voltage sensor.

Voltage-gated ion channels – A static view of the voltage sensor in the activated state

Voltage-gated ion channels form the third largest superfamily of signal-transduction proteins with 143 members in the human genome [11]. In this family classical voltage-gated Na, Ca, and K channels are found together with for instance Ca^{2+}-activated, cyclic nucleotide-gated,

and hyperpolarization-activated channels. All these channels are composed of four subunits (or four linked domains as in Na and Ca channels) symmetrically arranged around a central ion-conducting pore. In addition a number of auxiliary subunits can co-assemble with the ion-conducting α subunit to form physiological ion channels. The focus of this review is on voltage-gated K (Kv) channels but the conclusions are also, most likely, applicable to voltage-gated Na and Ca channels. Each α subunit of the Kv channel contains six transmembrane segments named S1 to S6 and has a modular organization (Fig. 1A). The intracellular N and C termini are important for fast inactivation, tetramerization of the channel, and regulation by Ca^{2+} and cyclic nucleotides. Structural details of these intracellular parts will not be considered here. The remaining six transmembrane segments consist of two distinct modules: the pore domain (S5-S6) and the voltage-sensor domain (VSD; S1-S4). Fig. 1B shows an ion channel tetramer.

The pore-forming unit conducts ions

Four pore domains make up the pore-forming unit through which ions will pass once the channel opens. The linker between S5 and S6 forms a narrow pathway, the selectivity filter, which determines which ion can pass through the pore. At the intracellular end of the pore-forming unit the four S6 helices form a vestibule with a narrow entrance that prevents ion flow in the channel's resting (closed) state but allows conductance of ions when the channel is activated (Fig. 1C). This narrow entrance is called the internal gate. The pore-forming unit was first identified when an inward rectifier channel was cloned [12], which is a channel built-up of isolated pore domains lacking the VSD. The structure of this central part of the channel has been described at atomic resolution during the last 10 years starting with X-ray structuring and then molecular dynamics simulations and mutational approaches [4, 13, 14]. However, a remarkable structural prediction of the pore-forming unit appeared already in 1995 [15]. Thirty of the 143 channels in the superfamily of "voltage-gated" ion channels are

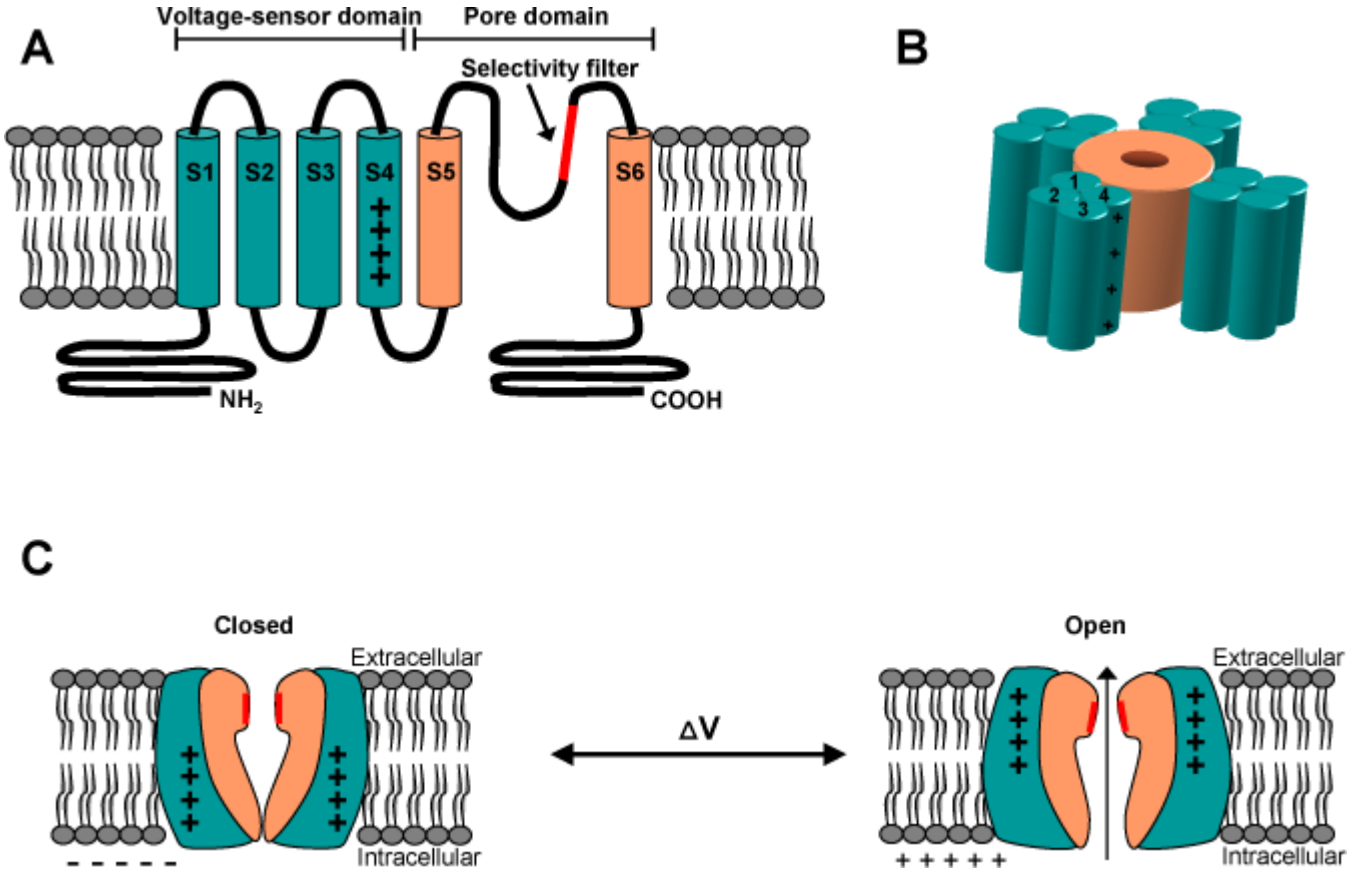

Figure 1: General architecture of a voltage-gated ion channel. (A) Each subunit is composed of six transmembrane helices named S1-S6 flanked by intracellular N and C termini. S1-S4 forms the voltage-sensor domain, VSD (green) with a positively charged S4, and S5-S6 forms the pore domain (orange) with the selectivity filter (red). (B) Four subunits tetramerize to form an ion channel with a central pore-forming unit (orange) surrounded by four VSDs (green). The intracellular N and C termini are removed for clarity. (C) A change in membrane voltage moves S4 charges in outward direction leading to the opening of the ion channel.

only built up of pore domains [11]. The remaining group of 113 channels also includes the VSD.

The voltage-sensing domain is a domain on its own

Via the S4-S5 linker helix the S6 gate is coupled to the four VSDs located outside the pore-forming unit. This coupling enables channel opening and closing upon changes in the membrane electric field. Most channels open at positive voltages but there are also channels that open at negative voltages like the hyperpolarization-activated HCN channels. It is the nature of the coupling between the gate and the VSD that decides if the channel will open at positive or negative voltages [16]. Since the pore-forming unit can exist on its own, it had been speculated that the VSD evolved separately as an autonomous protein and only later connected to a pore-forming unit [17]. Early indications of an independent VSD was the successful crystallization of the VSD of the bacterial KvAP channel [18] and the finding that a VSD transferred to a voltage-independent ion channel also transferred the voltage sensitivity [19]. In 2005, Okamura and co-workers showed that the enzyme Ci-VSP from *Ciona intestinalis* (a sea squirt) contains a VSD that is coupled to a cytoplasmic phosphatase [20], instead of being coupled to an ion-conducting pore. As in voltage-gated ion channels, the VSD senses the transmembrane field and activates the phosphatase at depolarizing voltages [21]. This was the first example of an isolated VSD that responds to voltages and thereby regulates the activity of something else than an ion channel. This channel is suggested to work as a monomer [22]. Finally, in 2006, a channel composed of the isolated VSD without coupling to a pore-forming unit or an enzyme was found [23]. The same year, a human isoform was identified and named Hv1 [24]. This channel is selective to protons and is shown to work as a dimer [25-27]. For a review on VSD proteins, see [28]. These findings show that the VSD can work as a functional unit on its own. VSD-coupled enzymes and the VSD proton

channels will likely become important tools for studying the general principle of voltage gating, including the organization of the VSD, and the coupling between the VSD and pore-forming unit in voltage-gated ion channels.

Sequences of 27 VSDs are shown in Fig. 2A. As pointed out in previous work some residues are very well conserved among all voltage-gated ion channels [29, 30]. Fig. 2B shows the frequency of the most conserved residue in each position (open circles). The pattern of conservation follows a very clear, one-in-three-to-four, pattern (see asterisks below the sequences), which is what we expect for an α-helical secondary structure with one side conserved. S4 follows a one-in-three pattern. The numbers 1 to 5 below the arginines of the S4 sequences denote the position of the first five positive residues expected to be involved in voltage gating. These gating charges are hereafter referred to as R1, R2, R3, R4 and K5. The filled circles denote >85% conservation of a certain type (polar, negative, positive, aromatic, polar/negative). S1 has only two residues reaching >65% identity (dashed line) or >85% similarity (dotted line). In addition, a glutamate at the extracellular end is found in >50% of the VSDs. These three residues are located on one side of an α helix (see underlined asterisks below the sequences). S2 has four very well conserved residues. All are on one side of an α helix (see underlined asterisks). S3 has only one very conserved residue. The conserved residues in S1-S3 are located in the extracellular half of S1, all over S2, and in the intracellular end of S3 (see also the structure in Fig. 3). Notably, the conservation in S3 is very low in the C-terminal end (called S3b in [18]). S4 has four well conserved positive charges (underlined numbers below the sequences).

The atomic structure of the VSD in an activated state

The tentative structure of the VSD was explored in many investigations between 1993, when the VSD was found to be a separate domain from the pore [12], and 2003, when the first X-

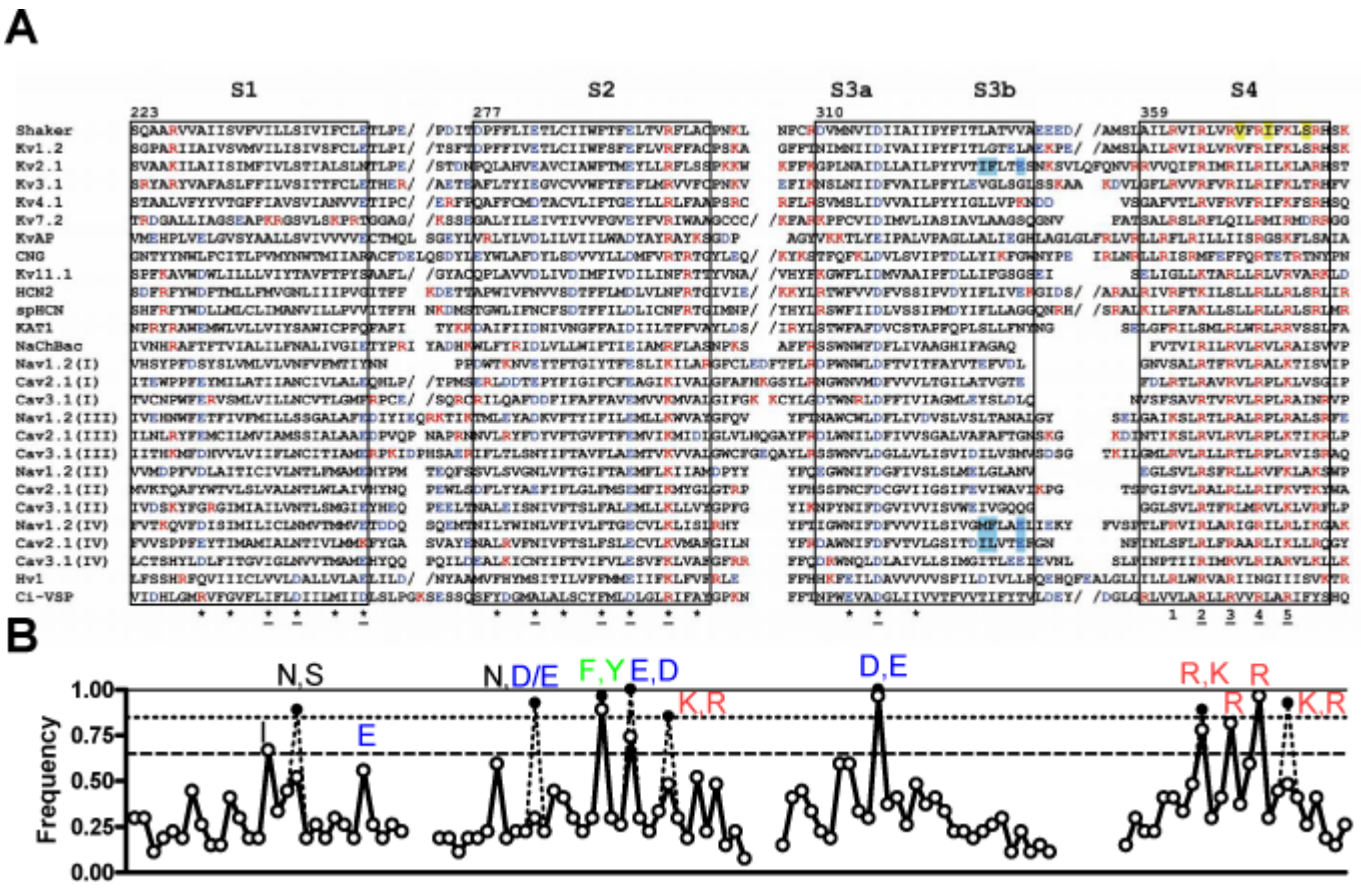

Figure 2: Conservation of residues in the voltage-sensor domain. (A) Sequences of 27 VSDs. Positively charged residues in red, negatively charged in blue, ILT mutation marked with yellow fields, and site for voltage-sensor trapping toxins marked with blue fields. Boxes denote the helical parts in the Kv1.2/2.1 structure [36] and the associated number on the top corresponds to the first residue in each helical part in the Shaker. / / means that >3 residues have been omitted. The asterisks below the sequences denote one side of an α helix and numbers 1-5 the first five gating charges in S4. Sequences from Shaker (P08510), rKv1.2 (NM_012970), rKv2.1 (NM_013186), rKv3.1 (NM_012856), hKv4.1 (NM_004979), mKv7.2 (Q9Z351, =mKQT2), KvAP (Q9YDF8), bCNG (NP_776703), hKv11.1 (Q12809, =hERG), mHCN2 (NM_008226), spHCN (Y16880), KAT1 (Q39128), NaChBac (AAR21291), rNav1.2 (P04775, =RNaBII), rCav2.1 (M64373, P/Q-type), rCav3.1 (O54898, T-type), hHv1 (NP_115745), Ci-VSP (NP_001128). S4 for KvAP has been shifted to let the structures of KvAP and Kv1.2/2.1 overlap [58]. (B) Frequency of the most conserved residues (open circles). If similar residues (polar, negative, positive, aromatic, and (in one case in S2) polar/charged) reach >85% (dotted line) this is denoted with closed circles. Conserved residues (>65 %, dashed line) are specified with amino acid identity in one-letter code. The first letter is the most conserved residue (open circle) and together with the second letter this makes up to the closed circle. Residues with >65 % identity or >85% similarity are marked with underlined asterisks below the sequences in (A).

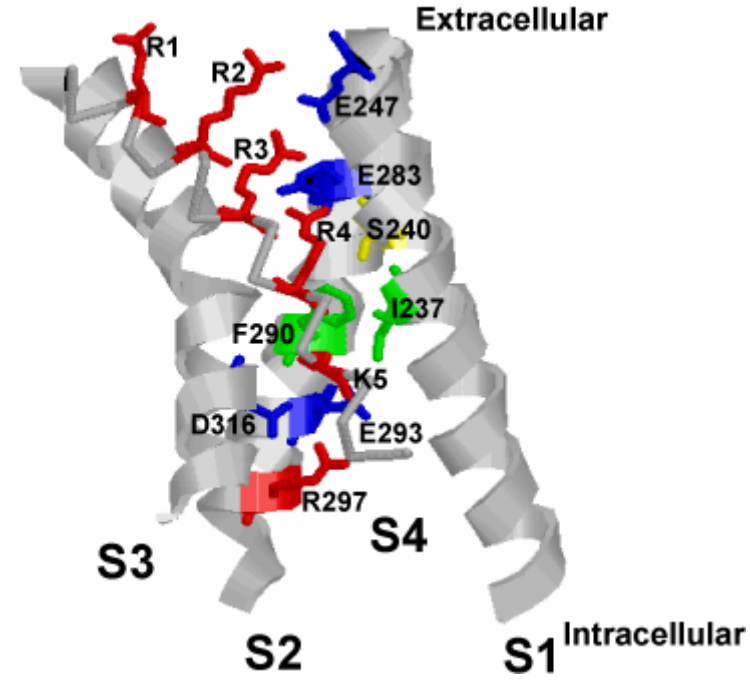

Figure 3: Conserved residues in S1-S3 (from Fig. 2B) encapsulate positive charges in S4 in the open state. Structure of Kv1.2/2.1 from [36]. Numbering for Shaker. Positive charges in red, negative in blue, hydrophobic in green, and polar in yellow. K5 is located in a cluster of charged residues (R240, E236, D259) below the hydrophobic residues (I237 and F290). R3 and R4 are located in a cluster of charged residues (E247, E283, R3, R4) above the hydrophobic residues.

ray structure of a VSD in isolation, cleaved off from KvAP, was published [18]. In several of these investigations, relatively accurate structures were developed [31-35]. Subsequent X-ray studies of mammalian Kv channels have confirmed and further refined these structures [36, 37]. Here we will use the most recent structure (a chimera between Kv1.2 and Kv2.1 which was crystallized without antibodies and in the presence of phospholipids at a resolution of 2.4 Å [36]) to discuss the architecture of the VSD in an activated (open or slow inactivated) state. In the following chapter, several functional studies will be included when discussing the voltage-sensing mechanism from a functional point of view. The VSD in the activated state is built up of S1 to S4 organized as anti-parallel helices (Fig. 3). All the conserved residues in S1-S3 are delineating a valley (the gating canal or gating pore), sheltering the central positive charges of S4. Much of the functional data are from the Shaker K channel from *Drosophila*

melanogaster. Therefore, in the remainder of this article we will use the Shaker numbering of the amino acid residues.

Negatively charged residues make contact with positive charges in S4

S4 contains several positively charged residues (mainly arginines) of which the first four, R1 to R4, have been suggested to be most important for voltage sensing [38, 39]. The positive charges are found in every third position and are therefore restricted to one screwed side of S4 (Fig. 3). Negatively charged residues in S1-S3 interact with the positively charged residues of S4 within the membrane while the lipid head groups interact with those that are at the membrane surface [36, 40, 41]. This makes the interior of the channel protein neutral. Specifically there are two absolutely conserved negative charges (293 in S2 and 316 in S3) in Na, Ca and K channels [30] (see Fig. 2 and 3). Negative counter charges were early suggested to be important for gating [30, 32, 34, 42-45]. There is also a conserved positive charge in S2. However, mutating this residue does not affect gating but leads to low expression suggesting that this residue is important for proper folding and expression (A. Broomand and F. Elinder, unpublished data). In the activated state, R4 is close to the narrowest region of the hour-glass shaped gating pore. When mutated to a histidine, this residue allows proton transfer at positive voltages [46]. In proton channels, the homologous residue is an aspargine allowing transfer of protons at positive voltages [25].

Water filled crevices in the channel focus the electric field

The X-ray structure also shows deep crevices on both sides of the channel protein where water can penetrate close to the center of the VSD. The crevices arise from the VSD helices being tilted against each other, and S3 being bent in the middle at a relatively conserved proline leading to a separation in two α-helices (S3a and S3b). The central water-excluding portion of the VSD has been estimated to be only ~5 Å thick making a thin aperture for the

voltage sensor S4 to move through. The highly conserved phenylalanine in S2 (F290 in Fig. 3) forms such a barrier for the voltage-sensor charges [36, 47]. This makes the electric field very focused and puts larger forces on the gating charges. The focused field also decreases the amount of required negative counter charges [30, 48]. Water-filled crevices were first discussed in 1996 based on accessibility of methylthiosulfonate reagents [48-50]. Later on, other studies also suggested a thin water-excluding portion: 1) Under certain circumstances a single point mutation can lead to permeation through the voltage-sensor gating pore [46, 51, 52]. 2) Effects of ionic strength on gating currents suggest water-filled crevices [53]. 3) Fluorometric studies showed that the electric field is more focused than expected from the thickness of a lipid bilayer [54]. 4) Atomic-distance rulers of different lengths connected to cysteines in S4 have been interpreted to indicate that the transmembrane barrier for the membrane voltage drop is only ~4 Å [55]. However, alternative explanations for these data that allow thicker barriers have been proposed [47]. The water-filled crevices led to a suggested gating model (the transporter model discussed further in the next chapter) where S4 is only needed to rotate 180° around its length axis to transfer gating charges across the transmembrane voltage drop [56]. However, this bold model is not compatible with the relatively large translational movement shown in subsequent studies [57, 58]. Deep water-filled crevices are also found with EPR spectroscopy in an isolated VSD of KvAP showing that they are intrinsic to the VSD [59].

The VSD, including S4, makes important contact with the lipid bilayer

The crystal structures of the Kv1.2 channel [37] and the Kv1.2/2.1 chimera [36] show that lipids inside the membrane are in close contact with the VSDs, including S4 and the pore-forming unit. Earlier most investigators had placed S4 buried in the channel protein, in direct contact with the pore-forming unit, and shielded from the lipid bilayer by the surrounding S1 to S3 [35, 60, 61]. However, already in 2001, we predicted that one side of the S4 helix

should be in contact with the lipid bilayer [33]. We suggested that such an unorthodox solution allows large-scale movements of S4 with little friction from the fluid lipid bilayer. This lipid exposure was later on reinforced [62-65]. Furthermore, the close contact between S4 and the lipid bilayer opens up for a number of interesting possibilities: 1) S4 charges can make contact with negative charges of the phospholipids. In molecular dynamic simulations, the two outermost arginines of S4 (R1 and R2) in Kv1.2 establish salt bridges with the lipid head groups in the open state [40]. 2) Furthermore, studies on different Kv channels and the surrounding sphingomyelins [66, 67] and phospholipids [41] highlight the importance of the nearby charged head groups for proper channel function (see also the chapter "Modulation of voltage sensing" below). 3) Also, the close contact between the voltage sensor and the membrane may allow lipophilic substances to interact with and regulate the voltage-sensor activity of ion channels by acting from the membrane (see the chapter "Modulation of voltage sensing" below).

The VSD connects to the upper end of S5 in a neighbouring pore domain

Interactions between the VSD and the pore domain were first shown from electrostatic experiments and calculations, and fluorescence measurements [33, 68-70]. In subsequent studies based on disulfide linkage between S4 and S5 the VSD of one subunit was shown to make close contact with the pore domain of a neighbouring subunit, not with its own subunit [60, 71-74]. This proposed interaction between two neighbouring subunits was later on supported by the X-ray structure of Kv1.2 [37]. A possible reason for this arrangement is that the VSD comes in a better position to pull the gate in the pore-forming unit open.

Similarities and differences between VSD structures derived with different methods

Two atomic structures of the voltage sensor and its surrounding in voltage-gated ion channels are available, and both are considered to be in the activated configuration (thus either an open

or an inactivated state of the ion channel): The first structure contains only the isolated VSD of the bacterial KvAP channel [18]. The second is included in a complete Kv channel (the chimera between Kv1.2 and Kv2.1) [36]. (In addition, the atomic structure of the VSD of a voltage-independent channel was recently determined in a putative resting configuration [75] and will be discussed in the following chapter) The two structures have much in common. For instance, conserved negative charges in S2 and S3 are pointing towards conserved positive charges in S4. However, the four N-terminal positive charges in KvAP, suggested to be homologous to "R1"-R4 in the Kv1.2/2.1 chimera, are in very different positions in the two crystal structures. In recent electrostatic experiments on the Shaker K channel, we arrived at a solution consistent with the Kv1.2/2.1 structure [58]. An EPR study of KvAP suggests that the complete VSD of the Kv1.2/2.1 chimera must rotate 70-100° clockwise (viewed from the extracellular side) to obey the EPR data for the KvAP channel [59]. We do not know the reason for the discrepancy between the two structures, but the most likely explanation is that the four N-terminal charges are not homologous in the bacterial KvAP channel and the Kv1.2/2.1 channel from the animal kingdom.

Also, wild-type Kv1.2 along with its cytosolic β-subunit has been structurally determined, although at a lower resolution [37]. In a recent study, the structure of Kv1.2 was compared with experimental data where a metal ion bridge was created between R1 in S4 and the extracellular end of S5 [76]. From molecular dynamics simulations constrained with experimental data it was concluded that S4 of the X-ray structure should be shifted 7-8 Å and rotated 37° counter-clockwise (viewed from the extracellular side). A similar shift in the same direction was also found for an unbiased molecular-dynamics study [40] and when comparing the Kv1.2 structure with the Kv1.2/2.1 chimera structure [36]. Taken together, this suggests that Kv1.2 and Kv1.2/2.1, not unexpectedly, probably have similar structures in a lipid

bilayer, and that the Kv1.2/2.1 crystal structure is more representative of a native channel conformation than the Kv1.2 crystal structure.

Notably, in a recent study with the Shaker K channel, we could not make disulfide bonds between inserted cysteins in S3b and S4 pointing towards each other in the Kv1.2/2.1 chimera, while we succeeded to make bonds between residues not pointing towards each other [58]. Thus, S4 in the native Shaker K channel is rotated relative S3b compared to the Kv1.2/2.1 crystal structure. This suggests, most probably, that the Kv1.2/2.1 crystal structure is not in a native form with respect to the S3b-S4 relation.

So far, we have considered the static VSD in the activated state. To close the channel, the voltage sensor must move to a resting state. In the following chapter we will discuss possible models for this voltage-sensor movement.

Gating charges cross the membrane electric field – A dynamic view of the voltage sensor

The voltage-gated ion channel described above is caught in an activated state. A voltage-gated ion channel has never been trapped in a resting (closed) state with X-ray crystallography. Recently however, a closed state of the non-voltage-gated six-transmembrane MlotiK1 channel was structurally determined [75]. In contrast to the voltage-gated ion channels, this channel lacks the positive gating charges R1-R4 and two of the negative counter charges, and shows a more compact S1-S4 arrangement. Because the entire S1-S4 domain is suggested to move as a rigid body during channel activation we will not include the structural information from the MlotiK1 channel in this section. Here, we will present information about a possible resting conformation of a VSD. We will also review some molecular details about voltage-

sensor movement – some of which is based upon consensus in the field of ion channel physiology and some of which depends on a diversity of ideas. But first we will recapitulate some basics of channel voltage gating.

The voltage sensor moves charges across the membrane electric field

Voltage-gated ion channels are extremely sensitive to changes in the transmembrane voltage. At relatively negative voltages (−100 to −60 mV), the open probability changes 10 times every 5 mV for a Na or a K channel [38, 77]. This is about 12 times more sensitive than an electronic transistor gated by one elementary charge [78] and depends on the design of the VSD. Already in 1952, Hodgkin and Huxley proposed that charge-containing particles sense changes in the membrane electric field, and that the movement of these voltage sensors open the channel [79]. Since then, the positively charged S4 has been identified as the voltage sensor [30, 78, 80-82]. At negative membrane voltages, S4 is located in a down position closer to the intracellular side of the channel. Switching to positive membrane potentials drives S4 in an outward direction through the membrane towards the extracellular side of the channel. This movement opens the inner gate of the pore-forming unit which allows ions to pass through the channel (see Fig. 1C). The voltage sensitivity discussed above suggests that at least 12 charges are needed to move through the entire electric field to open a standard ion channel. The transmembrane movement of the positive charges generates gating currents [83, 84]. By measuring the total gating charge and the number of channels in a membrane preparation, the number of gating charges per channel can be calculated. In the voltage-gated Shaker K channel about 13 e_0 charges per channel move during activation [38, 39, 85]. In Na channels about 12 charges are transferred [77, 86], and in Ca channels about 9 charges are transferred [87]. All these studies together suggest that about 12 charges are needed to open a channel and that there are no non-functional gating charges. Because there are four subunits

per channel, about three charges per subunit are needed to move through the entire electric field to open the channel.

It is generally agreed on that the activation can be divided in two main components: i) independent outward movements of the four S4 helices, followed by ii) a concerted opening step when probably all S4s move together [88-92]. However, the exact movement of S4 and its relation to the other helices in the VSD is still highly debated. The main original theories will be discussed in the following section.

Three major schools - how to move charges through the membrane

During the years since Hodgkin and Huxley's 1952 papers several ideas have been proposed regarding how gating charges move. Sparked by the first crystallization of a voltage-gated K channel, KvAP [18, 62], the controversy has even escalated [93]. Three conceptual models have played the major roles in this drama (Fig. 4):

1) Following the first cloning of a voltage-gated ion channel [82], the *helical-screw or sliding helix* model was first suggested in 1986 [42, 43]. This model suggests that the positive charges in S4 make contact with negative counter charges in other channel parts. When the membrane potential is changed, S4 moves 4.5 Å and rotates 60° along its length axis to make new contacts with the negative charges. To transfer the three charges per subunit, S4 has to move three steps, that is translate 13.5 Å and rotate 180° [30, 35, 44]. Several researchers have used the helical-screw model to explain their data [32, 34, 70, 86, 94-97]. The helical-screw model shares commonality with the pre-cloning model suggested by Armstrong [98].

2) Based on the water-filled crevices discussed above, a model with a large rotation but without a significant translational movement was suggested in 1997 [56]. It has received

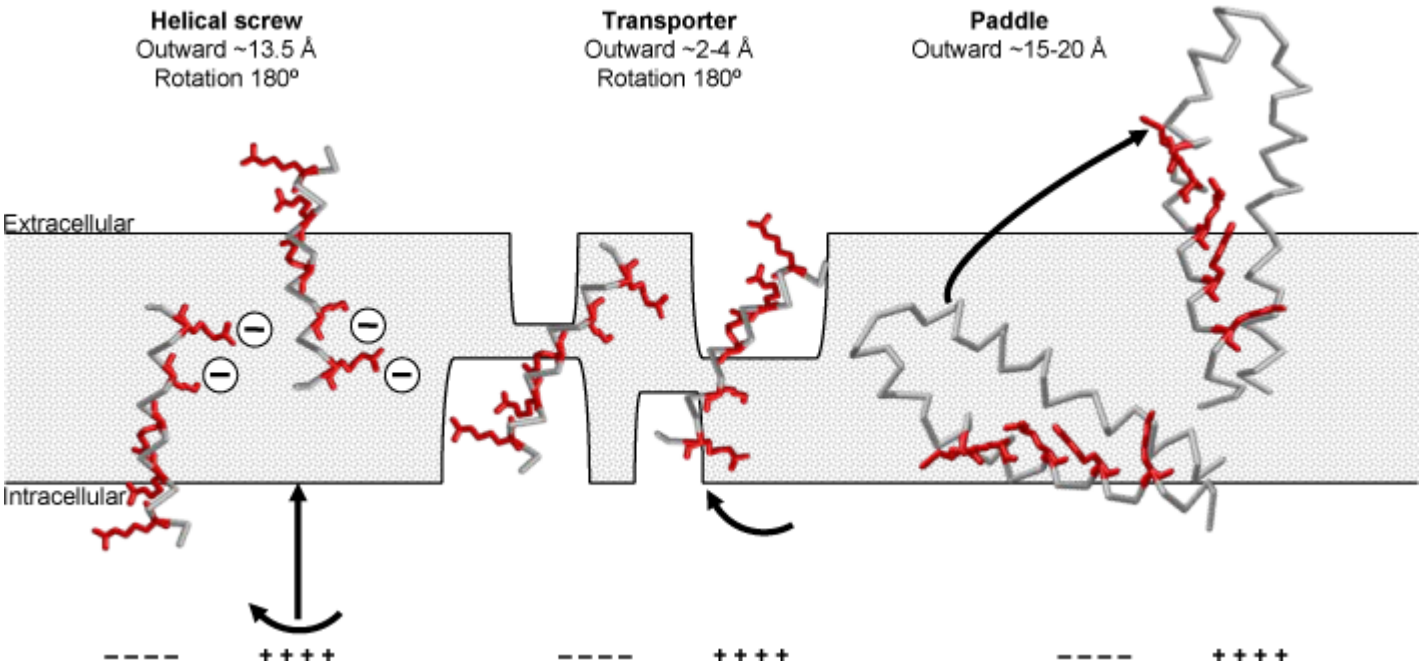

Figure 4: Three models for how S4 moves during opening. The negative signs in the helical screw model represent counter charges in S2 and S3.

many labels like *transporter-like, helical-twist, helical-tilt, or rocking-banana* models [56, 99-101]. The idea is that mainly rotation, and not very much translational movement (2-4 Å), is needed to transfer charges from the intracellular to the extracellular solution [101-104].

3) Based on X-ray crystallography data, the *paddle* model was suggested in 2003 [62]. The major idea is that S4 and S3b (together called the voltage-sensor paddle) are in close contact and never leave each other during a complete gating cycle. In its original version, the paddle was moving relatively freely in the lipid bilayer like a hydrophobic cation, but in subsequent versions the paddle is moving tighter to S1 and S2 [36, 105]. The movement of the paddle is more extensive (15-20 Å) [57, 62].

All models have had their proponents and opponents. When the transporter model seriously entered the scene [101] it was soon criticized [44] but it has been the paddle model in its different versions that has been challenged the most in a two-front war [51, 58, 60, 72, 73, 93, 106, 107]. However, during the last couple of years the three models have approached each other [36, 47, 97, 108]: In all of these convergent models S4 rotates while translating 6-15 Å and the positive charges make contact with negative charges in S2 and S3. A more detailed development from the original conceptual models to the more contemporary models is discussed in Fig. 4 of Tombola et al. [10]. One aspect that still differs between the models is that in the paddle model, S4 is carrying S3b as cargo on its back. This will be developed and discussed in the following section.

Long-distance helical-screw motion of S4 on its way through the membrane electric field

Because no high-resolution structure exists of a voltage-gated ion channel in a closed state, the current view of the movement from the open state to the closed state stems primarily from

an extensive amount of functional data and molecular dynamics studies. Recently, several suggestions have been published about the resting state of the voltage sensor [36, 58, 94, 96, 97, 108, 109]. Here, we will present a possible resting state and discuss some components of the S4 movement together with previous findings.

Resting state coordinates and the consequent activation movement

While the open state is evident from X-ray crystallography [36], the closed state is less defined. However, several recent investigations give some interaction coordinates. A histidine mutation of R1 gives rise to a proton current at negative resting voltages [51]. This suggests that the histidine is located in the narrow part of the short gating pore where S4 is moving. Because, as mentioned for the open state above, a histidine mutation of R4 gives rise to a proton current at positive voltages [46] (see Fig. 3), it is difficult not to imagine that R1 in the closed state is in the same position as R4 in the open state thus supporting a 180º rotation and 13.5 Å translation during activation. A mutation of the same top-charge residue (R1) to smaller residues gives rise to a cation current (called the ω current) at negative voltages [52], suggesting that a plug (the longer and charged arginine chain) is removed from the thin part of the channel protein separating the intra- and extracellular solutions. E283 (the top negative charge in S2) is close to the narrow part of the ω pathway, suggesting that R1 and E283 are close to each other in the deepest resting state [52]. ω-like currents have also been reported for the Nav1.2 channel [110]. However, in this case, two residues in domain II had to be mutated (R2Q and R3Q, see Fig. 2) to give rise to the ω current in the resting state. In addition, the double mutant R3Q/R4Q induced an ω current at positive voltages. One possible interpretation is that S4 in domain II only activates in two steps. Recently, mutations in R1 and R2 causing hypokalaemic periodic paralysis were shown to induce an ω current and this is probably a widespread mechanism causing disease [111, 112].

Further constraints for the down state come from conditional lethal/second-site suppressor yeast screens of the KAT1 channel [109], from proton-pore histidine mutations in S1 and S2 of Shaker [97], and from engineered disulfide bonds in Kv7.1 [113]. In the Shaker study, disulfide bonds could be made in the closed state between R1 and either I241C in S1 or I287C in S2. In the open state R4 is close to I241 and I287 (Fig. 3), suggesting that S4 must rotate 180º and translate ~13 Å to let R1 match up with I241 and I287 in the resting state. In the Kv7.1 study, "R0", that is three residues above R1, interacts with a residue corresponding to I241 in S1 in Shaker in the closed state. In the open state however, R1 instead interacts with I241, but in a suggested adjacent subunit. Structural modelling predicts that S4 rotates ~190° and translates ~12 Å accompanied by VSD rocking. This model also explains why the residue just before R1 can make a disulfide bond with itself in another subunit [71, 113, 114]. In a recent study we found that four residues in S4 (360, 363, 366, 369) can all make disulfide bonds with a residue in S3b (325) close to the conserved proline [58]. The bond formation is state dependent supporting a helical-screw movement with 9-13.5 Å translation and 120-180º rotation.

Based on the studies discussed above we propose a schematic model for the closed state of the channel shown in Fig 5A. When S4 is in the resting position, R1 contacts the top negative charge in S2 (E283), and R2 contacts the lower negative charge in S2 (E293) and the negative charge in S3a (D316). Residue I360 (the red dot at the external end of S4) is close to I325 (red dot at the N-terminal end of S3b). V363 (second red dot in S4) is also close to I325 in S3b in a closed state. Upon depolarization, S4 moves ~13 Å in the outward direction and simultaneously rotates ~180º to reach the activated state (Fig. 5B). Now R1 and R2 contacts the negative charge of phospholipids, R3 and R4 contacts E283 in S2 and E247 in S1 (see Fig. 3), and K5 contacts E293 and D316. In the open state (or last closed state), L366 (third red dot

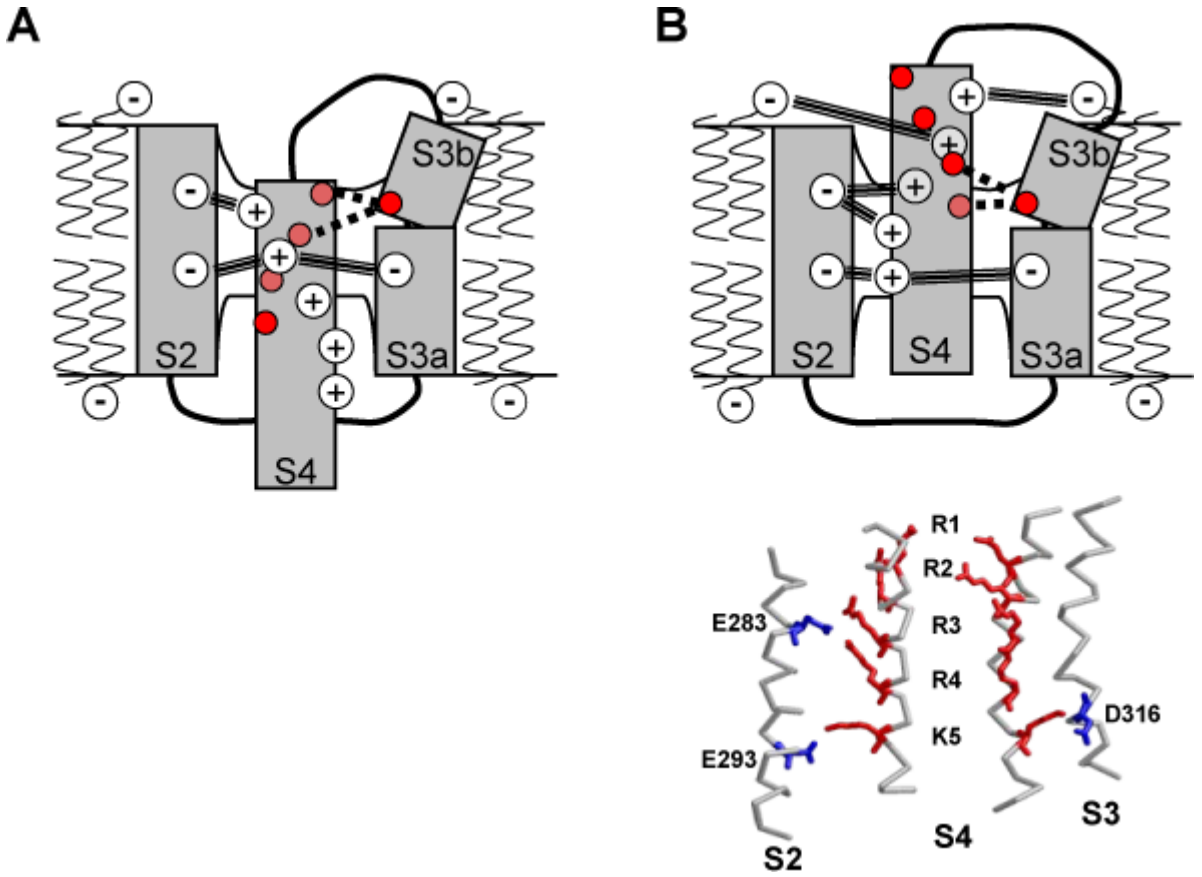

Figure 5: A schematic model for S2-S4 in closed and open configuration. (A) In the closed state, gating charges in S4 are stabilized by negative counter charges in S2 and S3 and the triple lines indicate electrostatic interactions. The red dots denote residues mutated to cysteines and the dotted lines denote disulfide bridges [58]. (B) During depolarization S4 moves ~13 Å outwards and rotates 180º to form new interactions in the open state shown. The interactions in the Kv1.2/2.1 structure are shown below.

in S4) and V369 (fourth red dot in S4) are close to I325. Thus, S4 moves relative to S3b. In the activated state the outermost gating charges seem to interact with and possibly be stabilized by surrounding lipid head groups. The proposed salt-bridge interactions are relatively similar to what has been proposed in several other studies [31, 45, 95, 115].

Further evidence for a long translational movement and 180º rotation of S4

As mentioned above, the translational movement of S4 from the closed to the open state varies from 0 to 20 Å in different investigations and models. While the translational movement has been debated for several years, there exists an almost general agreement of a large rotation (up to 180°) of S4 around its length axis [94, 101, 102]. The helical-screw and transporter models have rotation as a key element. In its most current version, the paddle model also includes rotation [36], even though part of the movement is suggested to represent translation without rotation because of the 3,10-helix pattern. Recently, based on the 3,10-helix pattern, a modified helical-screw motion was proposed where S4, except from undergoing a rotational and translational movement, also can elongate and stretch back when transitioning between an α-helical and 3,10-helical conformation [47]. This was based on the finding that S4 in both the activated Kv1.2/2.1 chimera [36] and the resting non-voltage-dependent MlotiK1 [75] partly adopt a rare long 3,10 helix. In the model suggested in Fig. 5 where S4 moves in three steps in an α-helical-screw fashion the translational movement adds up to 13.5 Å and the rotation adds up to 180°. If S4 is also tilting during translation, the size of the movement varies depending on where the translation is measured. Several other models concur. Tombola and collaborators suggest 10-13 Å and 180° rotation [94] and Pathak and collaborators suggest 6-8 Å translation and 180° rotation [108]. In general, these relatively large shifts are supported by accessibility measurements of cysteine-labelled residues measured in the open and the closed state [16, 48, 49, 116]. From electrostatic experiments we

suggested that R1 moves >12 Å towards the pore domain during activation [69]. A strong argument for a large movement normal to the cellular membrane comes from measurements of avidin accessibility to different-length tethered biotin reagents in KvAP [57]. A movement of 15-20 Å was found. However, these very large distances may not represent average movements but rather the distances of the channel protein caught in its most extreme thermodynamic fluctuation. In a recent luminescence resonance energy transfer (LRET) study, S4 was shown to translate ~10 Å [117]. Thus, even though there is some variability between the mentioned models, a general agreement ends up at ~8-13 Å movement.

In contrast to this, a number of investigations have suggested a very small translational movement [55, 101, 103, 104, 118]. However, we believe that the quantitative evidence against a large movement is not very strong. Rather, most of the data is *compatible* with a small movement but does not rule out a large movement. For instance, an elegant study by Ahern and Horn [55] shows that the voltage drop across the membrane occurs over a very short distance. This implies that a movement <4 Å is needed to transfer one gating charge from the intracellular bulk potential to the extracellular bulk potential. This does not however, rule out a large movement. To transfer several equally spaced gating charges attached to S4 a much larger movement is needed. In LRET studies, only small changes in distances were measured between different S4s, or between S4 and the pore [101, 104]. It was suggested that this precludes a vertical movement of S4. However, energy-transfer studies give more weight to shorter distances, and because S4 is rotating at the same time, we think it is difficult to make any statement about the translational movement based on the limited data set. In an extensive follow-up study, the translational movement was found to be ~10 Å [117]. In another study, a spider toxin was found to bind to the voltage-sensor paddle (see also chapter about modulation below). The fact that, in this case, the relatively large toxin molecule will be

stuck to its position in the lipid bilayer has been taken as evidence for minimal, if not absent, vertical movement of the paddle [118]. However, as will be discussed in the following paragraph, a simple solution to this is that S4 and S3 move in relation to each other during gating. Thus, S3 could stay stable when the toxin is bound while S4 is moving.

Movement between S3 and S4

The major difference between the suggested models is that the paddle model assumes that S3b hangs on S4 when it is moving through the channel protein. There is no hard evidence for this assumption except that S3b and S4 are stuck together in all available crystal structures. In contrast, while S4 has been shown to move a considerable distance upon activation, when measured with accessibility of cysteine-specific reagents (see above), it has been shown that S3b is not moving during gating [73, 107, 119]. This suggests that S3 and S4 should move relative each other. Even stronger arguments for a separation of the building blocks of the voltage-sensor paddle during gating comes from two recent studies where relative motion between S4 and S3b was assessed by making and breaking engineered disulfide bonds [58], or by measuring relative LRET-determined movements [117]. These experiments suggest that S4 moves 9-13.5 Å along S3b, which is inconsistent with the paddle model.

In some channels, the S3-S4 linker is short (domain I and II of some Na channels; see Fig. 2). Experiments where the S3-S4 linker in the Shaker K channel has been shortened show that the linker can be as short as three amino acid residues without affecting the activation kinetics [120]. Even if the linker is completely removed the channel can still gate, but now the kinetics are slow and the amount of gating charges reduced to about 50% [120]. Prima facie, this contradicts a large-movement, helical-screw model and supports a short-movement transporter model or the paddle model where S4 is not moving relative to S3b. However, as mentioned in the section above, we have strong arguments that S3b and S4 move relative to

each other and computer modelling clearly shows that S4 can move a large distance even if the linker is only three residues long [95]. For the case with the completely removed linker, we suggest the following explanation to solve this apparent contradiction: the activated state is consistent with a very short linker (Fig. 3). To move S4 down, S3 has to bend at the proline in position 322. However, the movement may be restricted so that the most resting state cannot be reached. This explains the reduced gating charge mentioned above.

To summarize, experimental data support a helical-screw movement with up to 13.5 Å translational movement and 180° rotation. In the following chapter we will discuss different ways to modulate the voltage-sensing mechanism.

Modulation of voltage sensing

Archetypical medical drugs like local anaesthetics and neurotoxins like tetrodotoxin from the pufferfish exert their effects by plugging the ion-conducting pore. However, another way to affect the number of open ion channels, and thus the functional output, is to affect the channels' voltage dependence. If the voltage dependence of a depolarization-activated channel is shifted in positive direction along the voltage axis, then the open probability (or conductance) is decreased, while a negative shift increases the open probability. Fig. 6 shows that, at voltages around −60 to −30 mV, a shift of the conductance vs. voltage, $G(V)$, curve with ± 4.2 mV is equivalent to increase or decrease the number of channels with a factor of 2 (see legend to Fig. 6). Thus, small changes in the channels' voltage dependence can have large effects on excitability (see chapter about excitability at the end of this review).

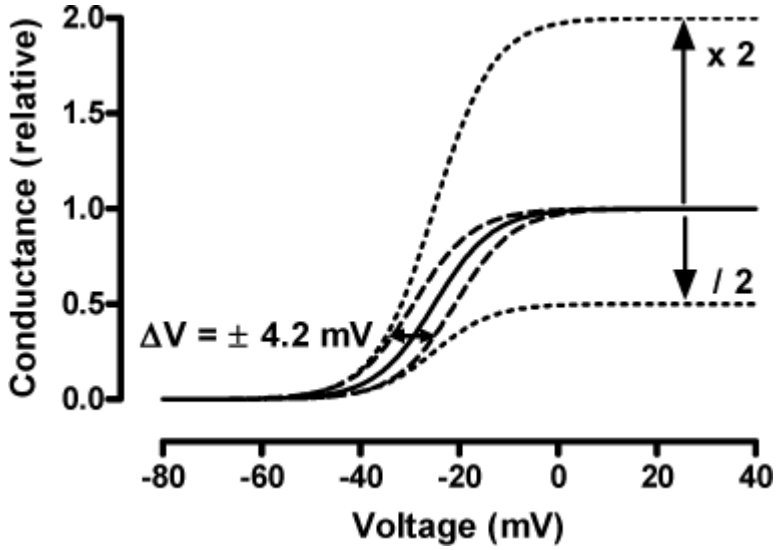

Figure 6: The relationship between voltage-dependence and the number of channels. The $G(V)$ curve is described as A / (1 + exp((V–$V_{½}$)/s)), where A is the amplitude of the curve, $V_{½}$ is the midpoint of the curve, and s is the slope factor of the curve. For the control curve (continuous line) A = 1, $V_{½}$ = –25 mV, and s = 6 mV. At negative voltages, where the curve approaches A exp(–(V–$V_{½}$)/s), the shift ΔV for a current change A is $\Delta V = -s \ln A$. A doubling of the amplitude of the curve is equivalent to shift the curve with –4.2 mV, and halving the amplitude is equivalent to shifting the curve with +4.2 mV.

Shifts can occur through a number of mechanisms. In some cases, there seems to be a direct interaction with the voltage sensor and in others, it is rather the gate that is affected. This review will mainly focus on the effects on the voltage sensor. We will divide this chapter into the effects from: 1) substances acting from the extracellular side, 2) substances acting from the intramembrane side, 3) substances acting from the intracellular side, and 4) direct (covalent) modification of the primary structure. Fig. 7 summarizes all types of effects.

Modifications from the extracellular side – Mainly direct effects on the voltage sensor

Ion channels' voltage dependence can be modified from the extracellular side by a number of freely moving molecules. Here, we will discuss metal ions, free fatty acids, toxins, and small-molecule channel openers. It should be noted that even though the substances are applied from the extracellular side, some of them bind to the lipid membrane and exert their effect

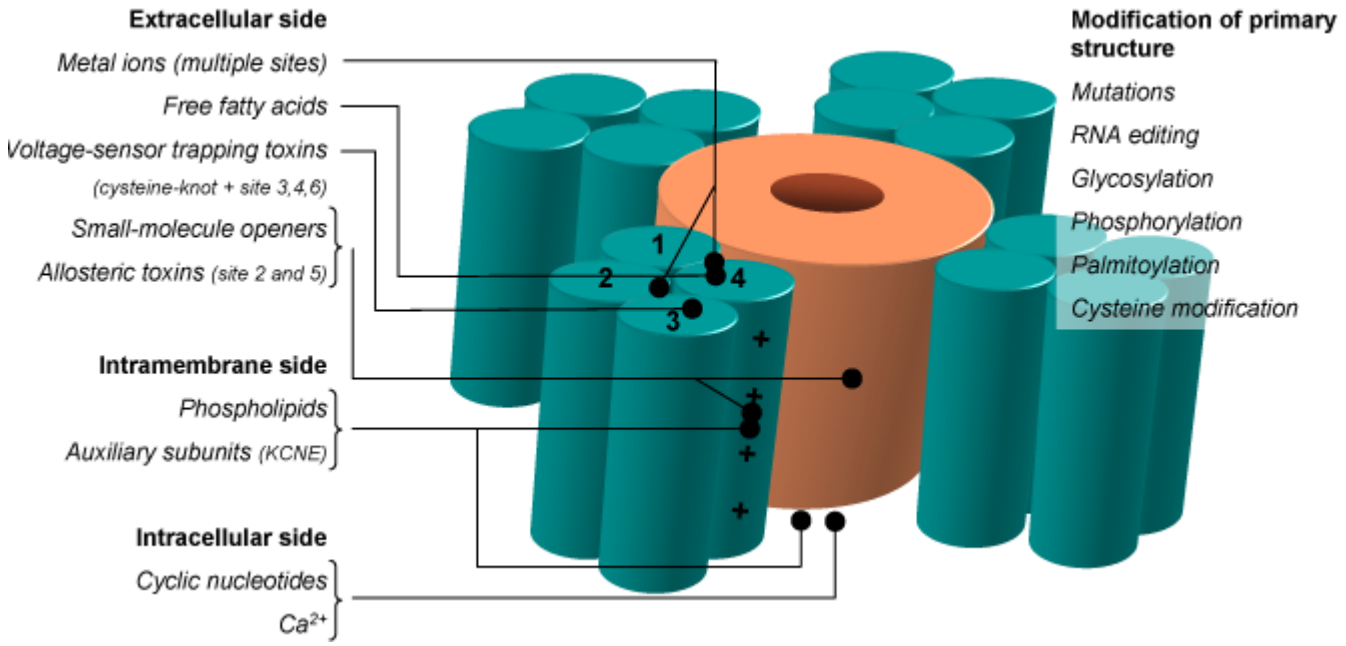

Figure 7: A schematic summary over modulation of the voltage sensitivity. The pore-forming unit is shown in orange and the four surrounding VDSs in green with S1-S4 labelled in one subunit. Note, that this is only a rough description of the interaction sites. See main text for greater detail.

from there, and some penetrate through the membrane and exert their effect from the intracellular side.

Metal ions

Besides binding to specific sites (as for the Ca^{2+}-binding site in BK channels, see "Modification from the intracellular side" below), metal ions can affect most voltage-gated ion channels by more general mechanisms [reviewed in 99]. Four main mechanisms have been discussed: 1) screening of fixed surface charges, 2) electrostatic effects from binding to the channel surface, 3) non-electrostatic effects from binding to the channel surface, and 4) block of the ion-conducting pore. Depending on the type of metal ion and the type of ion channel, one or several of these mechanisms can be dominant. For instance, for Kv channels, group 2 metal ions like Mg^{2+} and Ca^{2+} act primarily through a surface-screening mechanism in which they shield fixed surface charges by acting from the solution. This results in a $G(V)$ shift in depolarizing direction. These ions are therefore suitable tools for studying surface-charge effects. Transition metals such as Ni^{2+}, Cu^{2+}, and Zn^{2+} also affect the activation and deactivation kinetics, and lanthanides in addition block the pore at relatively low concentrations demonstrating the presence of mechanisms 2-4 for some metal ions. Ether-à-go-go (EAG) K channels seem to have a more pronounced sensitivity to metal ions. For instance, 2 mM extracellular Mg^{2+} slows the activation about 30-fold [121]. The reason for this high sensitivity is that the metal ions bind to an extracellular-facing crevice between S2 and S3 lined by negative charges specific for EAG channels [65, 122].

Free fatty acids

Charged lipophilic substances could act by incorporating into the membrane close to the channel or into hydrophobic pockets on the channel protein itself and then interact directly or electrostatically with the channel. One such group of candidate substances is the

polyunsaturated fatty acids (PUFAs) with a highly lipophilic acyl tail with two or more double bonds and a negatively charged carboxyl head group. PUFAs have been shown to shift the voltage-dependence of activation and/or inactivation of a number of different voltage-gated ion channels [123-128] suggesting a general and relatively unspecific mechanism of action. Direct PUFA-binding to the channel protein as well as PUFA-induced changes in the properties of the membrane have been suggested. However, from studies on the Shaker K channel we propose another mechanism which we call the *lipoelectric mechanism* [129]. By testing a number of different fatty acids we could identify that at least two double bonds in *cis* geometry together with the negative charge of the carboxyl group are needed to shift the voltage-dependence in hyperpolarizing direction [128, 129]. Interestingly, the efficacy of PUFAs is pH dependent with increased potency with increasing pH. The dramatic increase in current and corresponding shift of $G(V)$ from the PUFA docosahexaenoic acid (DHA) at pH 9 is shown in Fig. 8A-B. Thus, channels with different surface-charge profiles (and thereby different local pH) are differently sensitive to PUFAs. The lipoelectric mechanism is illustrated in Fig. 8C, and could be general for several charged lipophilic substances.

Toxins

Voltage-gated ion channels are specific targets for a number of toxins produced by plants and animals to defend themselves or to attack a prey. Toxins are also useful tools for studying channel structure and function. The mechanisms and sites of action are perhaps best known for Na channels where six main sites of interaction for neurotoxins have been identified [reviewed in 130]. However, some of these sites also have molecular functional correlates in other voltage-gated ion channels. In general, there seem to be three major mechanisms of action:

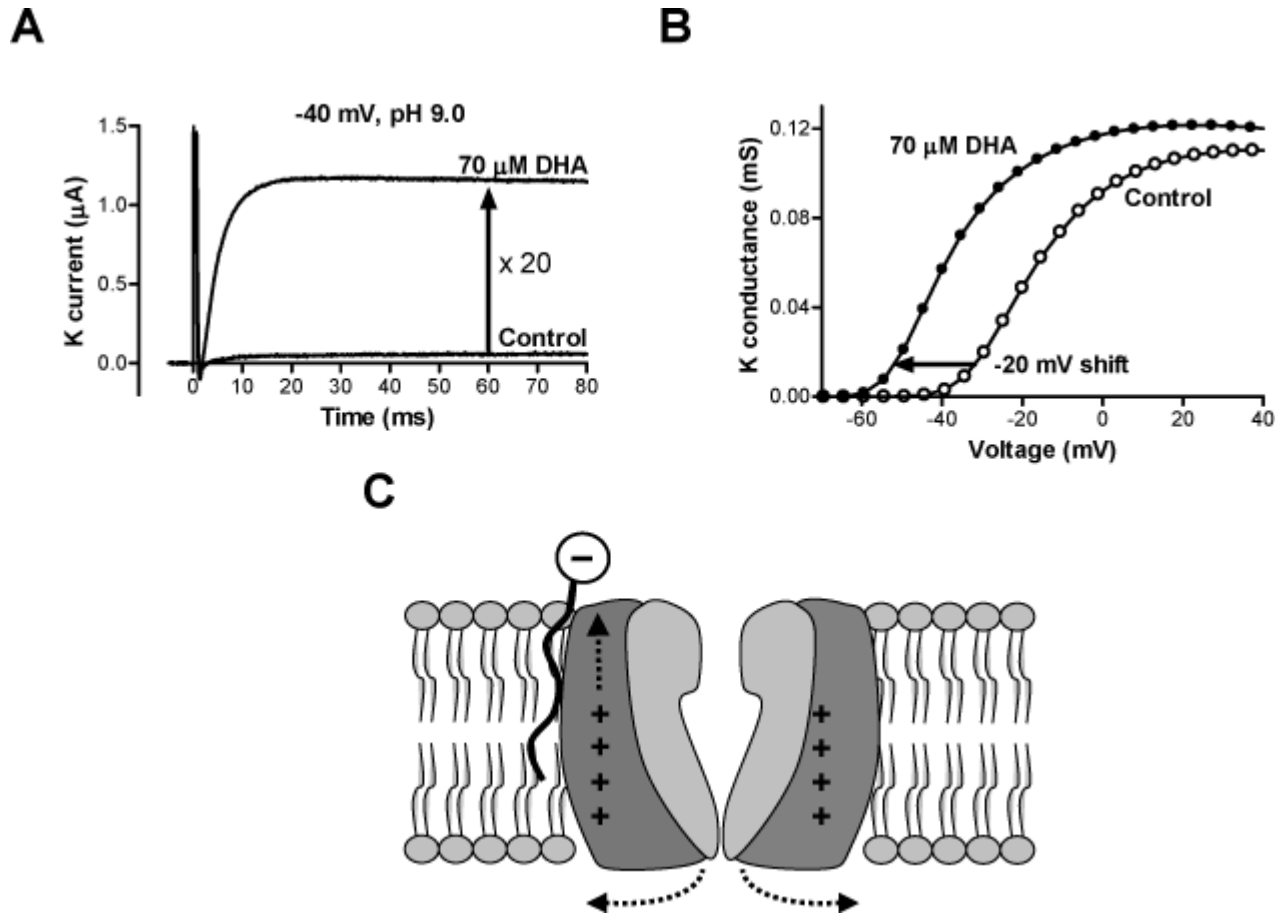

Figure 8: The effect of the polyunsaturated fatty acid DHA on the Shaker K channel (data from [129]). (A) 70 μM DHA at pH 9.0 induces a 20-fold increase in the current at –40 mV. (B) 70 μM DHA at pH 9.0 shifts the *G*(*V*) curve with –20 mV. (C) The proposed lipoelectric model [129] suggests that polyunsaturated fatty acids incorporate into the membrane or a hydrophobic environment close to the voltage sensor and electrostatically induce channel opening.

(a) Pore block is exerted by site-1 toxins which bind to the extracellular entrance of the ion-conducting pore. For Na channels, the typical examples are heterocyclic guanidines, tetrodotoxin (TTX), and saxitoxin (STX), and the peptide μ-conotoxin. For K channels, the peptide charybdotoxin, which also plug the pore, is probably homologous to site-1 toxins. In the present review, we are interested in toxins affecting voltage gating, and will therefore not discuss site-1 toxins further.

(b) Allosteric modification of many channel properties, such as single-channel conductance and gating, by binding to an intramembrane site close to the gate is exerted by site-2 and site-5 toxins. These are hydrophobic alkaloid toxins and other lipid-soluble toxins. Site 2 is located in S6 in domain I [131], separate from both the ion-conducting pore and the VSD. Binding to this site increases activation by favouring the open state of the channel combined with a prevention of inactivation. One toxin belonging to this group is batrachotoxin from the arrow-poison frogs (but also from some passerine birds). Batrachotoxin could possibly alter the movement of the adjacent S4 through indirect interactions upon binding to S6. Other site-2 toxins are the plant toxins veratridine, grayanotoxin and aconitine. A very similar effect to site-2 toxins is found for the site-5 toxins brevetoxin and ciguatoxin which also interact with S6 in domain I and in addition S5 in domain IV. To our knowledge, site-2 and site-5 toxins seem to be unique for Na channels and will not be discussed further here. However, binding from the membrane side is not only restricted to Na channels or to site-2 and 5 toxins. For instance amphipathic spider toxins bind to K channels via the lipid membrane as will be discussed below. Furthermore, the antiviral substance rimantadine was recently shown to bind from an intramembrane position to the gate of the M2 proton channel of influenza A virus to keep the gate shut [132]. As pointed out by these authors, the membrane-side binding inhibition could be advantageous for drug design because drug molecules are typically much

larger than hydrated ions selected by ion channels, and therefore the energy barrier for the drug to find a blocking site inside the channel pore would be much higher than targeting a functional site from the membrane side of the channel.

(c) Voltage-sensor trapping is exerted by site-3, site-4, site-6, and cysteine-knot toxins. Voltage-sensor trapping toxins include several unrelated peptide toxins from different phyla that bind to the extracellular S3-S4 linker in Na, K, and Ca channels and thereby trap the voltage sensor in either resting or activated state [130, 133]. The toxins have three to four disulfide bonds to make a rigid toxin molecule. The voltage-sensor trapping toxins can be further subdivided into three main groups depending on the effects they induce:

(1) α-scorpion toxins, together with sea anemone toxins, the spider toxin δ-atrachotoxin, and the tarantula toxin JZTX-I [134], are site-3 toxins that bind to the S3-S4 loop in domain IV of Na channels [135] and thereby slow inactivation. The reason that trapping S4 in domain IV in a resting state slows inactivation is that outward movement of this S4 plays a critical role for fast inactivation [89, 136-138]. A similar mechanism is suggested for the site-6 δ-conotoxins that interact with the S4 segment of domain IV close to site 3.

(2) β-scorpion toxins are site-4 toxins that bind directly to the extracellular S1-S2 and S3-S4 loops of domain II of Na channels with highest affinity for the activated channel [139]. The toxins are proposed to trap S4 in the outward position and thereby stabilize the open state. Thus, the Na-channel activation is enhanced, and the $G(V)$ curve is shifted in negative direction along the voltage axis. A structural model suggests that a β toxin fits tightly into the crevice between the S1-S2 and S3-S4 helical hairpins in domain II [140]. Magitoxin 5 is a spider toxin that also binds to site 4 [141].

(3) Some toxins bind to the S3-S4 linker and prevent the outward movement of S4 and thereby instead shift the *G*(*V*) curve in positive direction along the voltage axis. The K channel tarantula-toxin hanatoxin is the most thoroughly studied toxin in this group. In 1997, hanatoxin was shown to interact with the channel's four VSDs and thereby dramatically shift the channel's voltage dependence towards more positive voltages [107, 133]. The channel can still activate but a stronger depolarizing pulse is needed to overcome the larger energy barrier. Critical residues for the toxin – channel interaction are located in the external part of S3 (see blue fields in Fig. 2) [reviewed in 133]. Additional studies support a direct interaction with the VSD [118] even though the exact mechanism for interaction is poorly understood. The effects of hanatoxin are not restricted to Kv channels. Hanatoxin also inhibits the proton current through Hv1, the ionic current from Hv1-Kv2.1 chimeras [142], and a Cav current [143] (see blue field in Fig. 2). This suggests a conserved voltage-sensing structure. Several other tarantula toxins are related to hanatoxin. This group of toxins is called cysteine-knot toxins because they have a cysteine-rich core with three disulfide bonds forming a characteristic structure [summarized in 133]. Also other cysteine-knot toxins act on the voltage sensors of Kv [142-146] as well as Nav and Cav channels [134, 143, 147-150] and several of them show a promiscuous behaviour. For instance, the tarantula toxins JZTX-I and III bind to both Nav (see above) and Kv channels [134, 145, 146, 149], ProTx-I interacts with Nav, Kv, and Cav channels, and ProTx-II interacts with Nav and Cav channels [148]. In addition, there are other unrelated toxins like sea-anemone toxins that affect Kv channels with a similar mechanism [151-153]. The amphipathic structure of the cysteine-knot toxins with a cluster of hydrophobic residues surrounded by polar residues [133] suggests that they are likely to partition into the membrane and possibly act on the VSD from there [64, 154-157]. The hydrophobic residues are dipping deep into the lipid bilayer while the charged residues are

facing the polar head groups of the lipid bilayer, thus anchoring the toxin in a certain position. It has been shown that hanatoxin forms a strong and stable complex with the VSD [118]. Thus, it does not leave the channel during activation. The lipid bilayer does not play an energetically dominant role for the interaction between the toxin and the channel, but the partitioning in the lipid bilayer is possibly necessary for the toxin to reach the voltage sensor. The mechanism by which these toxins aggravate activation is not known but it has been suggested that hanatoxin when binding to the channel pushes S3 towards S4 and thereby hindering S4 movement upon depolarization [reviewed in 158]. However, the number of positive charges on one side of hanatoxin is striking. The position of the critical glutamate in the hanatoxin-binding motif of Fig. 2 is at about the right depth to be accessed by the positively charged residues of the toxin. If residue R3 in the toxin electrostatically interacts with the critical glutamate [133], there are two other positive charges (K10 and K26) that possibly could come close to S4 and electrostatically counteract activation. This is reminiscent of the *lipoelectric mechanism* discussed above for free fatty acids.

Small-molecule K-channel openers

Small-molecule openers (in contrast to the larger toxin molecules weighing ~3-4 kDa) of ion channels are rare but medically interesting substances [159]. For KCNQ (Kv7) channels there exist some such molecules [reviewed in 160]. KCNQ2 and KCNQ3 together form an ion channel responsible for the M-current playing a key role in dampening neuronal excitability. Reduction of this current (for instance by channel mutations) causes hyperexcitability diseases such as epilepsy [benign familial neonatal convulsions: 161, 162], arrhythmia, and deafness. Opening of this channel would thus prevent disease. Retigabine is such a newly developed anti-epileptic drug [163, 164]. Acrylamide (S)-1 and meclofenamic acid also opens the channel [165, 166]. Another recently described substance, zinc pyrithione (ZnPy), also opens KCNQ channels [167]. However, the mechanisms are different and the sites of action differ.

Retigabine mainly shifts $G(V)$, while ZnPy both shifts $G(V)$ and increases the maximum conductance. Retigabine is thought to open the channel by binding to a hydrophobic pocket between S5 and S6 and interact with a conserved tryptophan in the lower part of S5 and thereby keep the gate open [168, 169]. ZnPy interacts with crucial residues in the outer end of S5 and the pore helix [167]. The effects of the two substances are additive [170]. Also the benzodiazepine R-L3 potentiates Kv7.1 by binding to specific sites on S5 and S6 [171]. However, even though these substances affect the channel's voltage dependence by shifting the $G(V)$, the effect is on the gate rather than on S4. Molecules activating hERG have also been found [172-176]. Furthermore, auxiliary subunits can also work as binding sites for some channel activators [177].

Modifications from the intramembrane side – Effects on the voltage sensor and the gate

Some molecules are located in the plasma membrane and affect the channels' functions from this position. One type of molecule is auxiliary subunits that coassemble with the ion-conducting channel molecules. But first, we will discuss the most obvious candidate – the lipid bilayer itself.

Phospholipids

In addition to lipid-partitioning molecules affecting the VSD by acting from the membrane, also the membrane lipids themselves interact with the channel protein. Contacts between S4 and the lipid bilayer were suggested early [33]. This is supported by an intimate contact between the VSD and phospholipids in the Kv1.2/2.1 chimera crystal structure [36] together with molecular dynamics studies on Kv1.2 [40] or an isolated S4 [178] showing favorable interactions between lipid head groups and R1 and R2 in S4 (Fig. 5B). Correctly charged lipids are important for proper channel function of several Kv channels [41, 66, 67]. For instance for KvAP, the negative charge of the lipid phosphate group appears to be crucial for

channel function and voltage-dependence possibly by working as counter charges for the positive arginines in S4 [41]. A similar line of reasoning applies also to other Kv channels [66, 67].

Perhaps the most studied phospholipid in this context is phosphatidylinositol 4,5-bisphosphate (PIP_2) which is mainly located in the cytoplasmic leaflet of the plasma membrane, and from there affects the activity of a number of ion channels and transporters [179]. The proposed mechanisms for several of the effects on ion channels share a general feature: PIP_2 incorporated in the membrane mediates its effect from there, by acting electrostatically on cytoplasmic regions of the channel protein. All Kv7 (=KCNQ) family members are sensitive to PIP_2 and several homo- and heteromers display no channel activity after PIP_2 depletion [180, 181]. PIP_2 is suggested to interact with a conserved histidine residue in the C terminus to trap this part of the channel in the voltage-activated state, and thereby favouring an open pore [180, 181]. PIP_2 electrostatically promotes HCN channel activity by shifting the voltage dependence in depolarizing direction [182].

Also other membrane properties such as membrane thickness, fluidity, tension, and curvature can affect the function of ion channels [reviewed in 183, 184]. Already in the late 1970s, hydrocarbon anaesthetics were suggested to affect Nav channels by increasing the membrane thickness [e.g. 185, 186]. It was even speculated that the Nav voltage sensor would be facing the lipid bilayer and therefore respond to changes in membrane thickness [185]. The importance of the membrane organization has been further explored, often by the use of gramicidin channels [187]. It is now clear that several membrane proteins including channels can respond to alterations in the physical properties of the membrane [183, 188]. For instance, an increase in membrane cholesterol shifts the voltage-dependence of Nav channels in a

positive direction possibly by changing the bilayer elasticity [189]. However, the exact mechanism for how voltage-gated channels sense changes in membrane properties is not clear.

Auxiliary subunits

The channel structure shown in Fig. 1B is sometimes referred to as the α subunit. Many channels can further co-assemble with additional auxiliary β subunits. Disturbed interaction with auxiliary subunits can cause pathologic conditions like cardiac arrhythmias, thus demonstrating their physiologic importance [reviewed in 190, 191]. A large arsenal of different auxiliary subunits interacts with the pore or the cytoplasmic domains of Kv channels to change their gating [191, 192], and one cytoplasmic β subunit has been speculated to interact directly with the VSD to link the redox chemistry of a cell to changes in membrane potential and vice versa [193]. Lately it has also been suggested that some transmembrane auxiliary subunits interact directly with the VSD. We will here exemplify this by discussing the effect of KCNE subunits on KCNQ channels.

KCNE peptides consist of a single transmembrane helix, opening up for direct VSD interactions within the membrane. Two well-known complexes of KCNQ1 (KCNQ1/KCNE1 and KCNQ1/KCNE3) clearly demonstrate the gating effect by auxiliary subunits. Association of KCNE1 with KCNQ1 slows the activation and deactivation kinetics and shifts the voltage-dependence ~20 mV in a positive direction [194, 195], while KCNE3 makes the channel almost voltage-independent and constitutively open [196]. KCNE1 is suggested to prevent S4 movement by trapping the VSD in the resting state [197] while KCNE3, on the contrary, stabilizes the activated state [197, 198]. Exactly how the trapping occurs in not known but a direct interaction with S4 could be possible because the inner part of the transmembrane KCNE1 helix is associated with the pore-forming unit while the external part is located at the

lipid-protein boundary [199] and is close enough to S4 to form a disulfide bond [197]. A KCNE binding pocket has been suggested between the VSDs of two adjacent subunits close to the pore domain of a third subunit [198, 200, 201]. Two KCNE1 subunits bind per channel [202, 203]. This interaction and the subsequent effects tend to be complex because KCNQ1 channels can interact not only with one type of KCNE protein but also with two different KCNE subtypes at the same time [e.g. 204, 205].

Also the transmembrane auxiliary subunit DPP6 (also known as DPPX) induces shifts in the voltage-dependence, in this case in a negative direction for the Kv4 channel [206, 207]. A direct effect on the VSD is suggested where DPP6 may destabilize the resting state and thereby promote the outward movement of the voltage sensor [208]. Another example, on the same theme but with a linked "subunit", is the large-conductance Ca^{2+}-activated K (BK) channels. This channel has an unusual transmembrane topology with a transmembrane segment preceding S1, S0. Interestingly, this additional transmembrane helix binds within the VSD between S2 and S3 from where it probably stabilizes the resting state of the VSD [209]. The auxiliary β1 subunit of BK channels alters the voltage-dependence by interacting with S0, S1 and S2 in the periphery of the VSD [210, 211].

Modifications from the intracellular side – Mainly effects on the gate

Intracellular regulation of ion channels offers a link between the cell's metabolic state and the channel's activity. The voltage sensitivity is affected by a number of molecules targeting the intracellular side. For instance, Ca^{2+} and cyclic nucleotides bind to specific pockets. They are believed not to act directly on the VSD but instead to alter the movement of the gate. Interestingly, some bound metal ions seem to act not on the gate but directly on the VSD by changing the intracellular electrical potential around S4. Below, both the effects on the gate and on the VSD will be discussed.

Binding of cyclic nucleotides and Ca^{2+} to specific domains

The cyclic nucleotide-gated (CNG) channels and hyperpolarization-activated cyclic nucleotide-modulated (HCN) channels are both gated by cyclic nucleotides [refs. 212, 213 for a review]. CNG channels are only weakly voltage-dependent and the binding of cyclic nucleotides increases the current dramatically. In contrast, HCN channels are activated both by membrane voltage and by cyclic nucleotides. Because HCN channels are activated by hyperpolarizing potentials, this gives an increased open probability at negative potentials associated with, for instance, accelerated heart rate. Binding of cyclic nucleotides to the C-terminal cyclic nucleotide-binding domain (CNBD) promotes the open state of both CNG and HCN channels through the C-linker that connects S6 with the CNBD [214]. It is suggested that the CNBD, when not binding cyclic nucleotides, inhibits channel gating by constraining S6 movement, and that the strain and inhibition is released when cyclic nucleotides are bound [215]. A similar model can also be applied to Ca^{2+}-regulation of BK channels. BK channels can activate in response to depolarizing potentials also in the absence of $Ca^{2+,}$ but Ca^{2+} binding to the C-terminal binding domain shifts activation in a hyperpolarizing direction. A spring-like model for channel activation upon Ca^{2+} binding has been proposed [216]: In the absence of $Ca^{2+,}$ the C-linker is relatively stretched and functions as a passive spring providing a closed pore. Possibly, the linker applies a force on S6 that prevents activation. The energy obtained from Ca^{2+} binding is used either to reduce the inhibiting force on S6 or for the spring to pull S6 into the open configuration. In addition to Ca^{2+}, Mg^{2+} can also affect BK gating possibly by binding to a low affinity site in the C terminus [217]. Similarly to Ca^{2+}, Mg^{2+} shifts the voltage dependence in hyperpolarizing direction [e.g. 218]. However, the mechanism seems to be another: bound Mg^{2+} is suggested to electrostatically repel one of the positively charged arginines in the C-terminal part of S4 [219, 220]. This shows that cytosolic domains can be close enough to the VSD to have an electrostatic impact.

Modification of the primary structure

The most stable modification of an ion channel is to change the amino acid sequence. This can be achieved both on the gene and at the RNA level through mutations and for instance RNA editing, respectively. Many channel mutations are associated with disease but can also give insight into ion channel physiology. The purpose of RNA editing is yet not clear. One role could be to provide diversified channels originating from the same gene but possessing slightly different properties. Furthermore, covalent modification of different amino acids can also affect the voltage dependence of the channel.

Mutations

A number of mutations in the channel protein affect voltage gating. The mutations can be located throughout the protein, and their effects and mechanisms of action are diverse. Charged residues of two types affect voltage gating. Either they are 1) located inside the voltage sensing machinery directly, interfering with the gating charges, or they are 2) located outside, causing an electrostatic effect through space. Several studies have focused on, and found large effects of mutations of positive charges in S4, and negative charges in S2 and S3 [45, 221-223]. Charges outside the voltage-sensing residues also affect the voltage-sensing mechanism. A charged residue on the surface of the channel protein will electrostatically affect the voltage sensor to open or to close the channel. A positive charge on the extracellular surface will repel the voltage sensor and close the channel. A negative charge will attract the voltage sensor and open the channel. The closer the charge is to the voltage sensor, the larger the effect is on the voltage dependence. This strategy has been used to map the location of the voltage sensor [33, 58, 68]. Even residues at some distance from the voltage sensor can electrostatically affect the voltage sensor via a reorientation of charged neighbouring residues [224].

In addition to charged residues, neutral ones can also have a large impact on the voltage sensing mechanism. Here, we highlight two neutral-to-neutral Shaker channel mutants – the ILT and the V2 mutants. For the ILT mutant, three non-basic residues in S4 are substituted for the amino acids found in the related Shaw channel at corresponding positions (see residues marked with yellow field in S4 for Shaker, Fig. 2) [225]. In the V2 mutant, one leucine in S4 (just outside the sequence in Fig. 2 in the S4-S5 linker) is substituted for valine [85]. Even though these substitutions are very conservative, the effect on activation is dramatic with altered kinetics and a large shift of the $G(V)$ curve in depolarizing direction. The ILT and V2 mutations isolate the final opening transition by stabilizing an intermediate closed state or destabilizing the open state [88, 92, 226]. Although the exact mechanisms for ILT and V2 are not known, both of them are useful tools for studies on the gating pathway where it may be desired to separate early transitions from late transitions.

RNA editing and alternative splicing

The sequence of an ion channel can be modulated on the RNA level through RNA editing and alternative splicing. This process is catalyzed by enzymes that convert adenosine to inosine that is read as guanosine during translation. Editing of some Kv channels has been observed [227-229] among which the squid SqKv1.1A channel has been carefully studied [230]. Invertebrates differ slightly from vertebrates by showing hyperediting, with a large fraction of adenosines in mRNA being edited. This is also true for SqKv1.1A. Interestingly, most of the edited positions in SqKv1.1A are located in the well-conserved tetramerization domain, T1, in the N terminus. Editing of these residues give channels with reduced expression, possibly by disturbing the ability to tetramerize. Perhaps more surprisingly, the voltage-dependence of activation is in some cases shifted in depolarizing direction. Similar effects were found when studying mutations in T1 in Kv1-type channels [231-233]. The role T1 plays for gating is not clear but these studies imply that channel rearrangement during gating is not restricted to the

transmembrane domains but also involves T1 [234]. Considering the effects from intracellular C-terminal Mg^{2+} binding, one could speculate that the T1 domain may also interact electrostatically with the VSD and that this interaction could be influenced by charge-altering substitutions. In addition to RNA editing, alternative splicing can also affect gating. One example is alternative splicing in the S3-S4 extracellular linker which influences kinetics of S4 movement of a Ca channel [235].

Glycosylation

Covalent attachments on the channel surface can induce a permanent change of gating. For instance, some Kv channels have a glycosylation site in the extracellular loop between S1 and S2 that can be post-translationally modified. To this site, carbohydrate structures capped with the negatively charged sialic acid can be coupled. Incorrect glycosylation can lead to severe disorders like cardiac arrhythmias [236]. A clear relationship between the amount of sialic acid and voltage-dependence of channel activation is seen [237-239]. When increasing the glycosylation state, activation is shifted in hyperpolarizing direction. Screening experiments with divalent cations suggests an electrostatic effect where the negatively charged sialic acid reduces the local surface potential experienced by the voltage sensor and thereby facilitates channel activation [237-239].

Phosphorylation

Many ion channels, including voltage-gated channels, are also affected by enzymatically regulated phosphorylation, and the effect depends on channel type and phosphorylation site [240, 241]. For instance the N and C termini of Kv2.1 have together over 100 serines, threonins and tyrosines that all theoretically could be phosphorylated [ref. 242 for a review on Kv2.1 phosphorylation]. In reality only a few of these seem to act as phosphorylation sites, the majority of which are located in the C terminus. Dephosphorylation of these sites

enhances channel activity [e.g. 243]. A strong correlation is seen between the degree of phosphorylation and voltage-dependence where channels with a low phosphorylation level activate at more negative potentials compared to more heavily phosphorylated channels [242]. This could be important for tuning channel activity during ischemia and hyperexcitable events such as epilepsy that, via an increase in intracellular Ca^{2+}, activate calcineurin that dephosphorylates Kv2.1 and thereby possibly suppress firing [244, 245]. The molecular mechanism by which phosphorylation affects channel gating is not clear, however a surface charge effect similar to that for glycosylation is suggested [246]. An increase in negative surface charges on the inside of the membrane close to the voltage sensor will demand a stronger depolarizing pulse to open the channel. Several other Kv channels also have phosphorylation sites and they are not only restricted to the C terminus but include both the N terminus and cytoplasmic facing loops [247-251]. Interestingly, a single threonin in the S2-S3 linker plays a crucial role in switching the Kv1.2 channel between two distinct gating modes with different gating kinetics and voltage-dependence [252]. The capacity to switch is abolished when draining the cell, suggesting interactions between an intracellular component and the threonin. However, neither phosphate groups nor PIP_2 affects the switching and so far the identity of the cytoplasmic component is unknown.

Palmitoylation

Intracellular attachment of some other chemical groups is also enzymatically regulated providing a dynamic modification of the voltage-dependence. For instance palmitic acid can be linked to a cysteine in the S2-S3 linker of Kv1.1, a process named palmitoylation [253]. If mutating the cysteine, and thereby preventing binding of palmitic acid, the activation curve is shifted dramatically in hyperpolarizing direction. Also Kv1.5 can be palmitoylated at a cytosolic cysteine, however this cysteine is located in the C terminus [254]. The exact role of

palmitoylation is unclear, but perhaps it provides a hydrophobic connection between the membrane and channel important for VSD arrangement and movement [253].

Cysteine modifying reagents

Both under experimental conditions and in normal physiology, channels can be modified by substances that interact with cysteines within the channel protein [255]. Two such substances are N-ethylmaleimide (NEM) and hydrogen peroxide that interact with KCNQ channels and induce an increase in current, together with a hyperpolarizing shift of activation [256-258]. For NEM it has been proposed that cysteines in the C terminus are important for the shift, while cysteines in S5 are important for the increase in current [257, 258]. For hydrogen peroxide, three cysteines in the cytoplasmic facing S2-S3 linker are critical [256]. Another substance, thimerosal, also modulates the gating of KCNQ but by interacting with a cysteine in the outer part of S3 [259].

Voltage-sensor effects on excitability

Toxins can have dramatic effects on a channel's voltage dependence and their impact on excitability is easily appreciated. In contrast, the physiological significance of the seemingly small effects on gating induced by some pharmacological substances or by some disease-associated mutations may be less clear. Here, we will show computer simulations of nerve activity and we will discuss possible effects on excitability by small effects on the voltage dependence of K channels.

Therapeutic concentrations of PUFAs representing the increased concentration in cerebrospinal fluid during epilepsy treatment with a fat-rich ketogenic diet shifts the

activation of the Shaker channel with only ~2 mV in hyperpolarizing direction [128]. Is this effect large enough to significantly affect excitability of neurons? We performed computer simulations on the well-known myelinated frog axon [128, 260]. Fig. 9A shows a train of action potentials induced by a steady stimulating pulse of 0.8 nA. By affecting the voltage dependence of the voltage-gated K channel with as little as –2 mV, this repetitive activity is completely abolished without affecting the first action potential (Fig. 9B). Thus a very small effect on a channel's voltage dependence can be an effective tool to prevent hyperexcitability as in epilepsy or cardiac arrhythmia. To explore this effect in more detail we also tested different combinations of Na and K channel densities, and different stimulating currents. In Fig. 9C, repetitive activity is found for combinations within the areas associated with different Na channel densities (shown as maximal conductances of 1.5, 2.0, 2.5, and 3.0 μS) for different combinations of stimulating current (y-axis) and maximal K conductance (x-axis). Fig. 9D shows that the areas contract markedly for a shift of the voltage dependence of the K channels with –2 mV. All areas are contracted by a factor of about 1.55 (dashed lines in Figure 9D). Thus, a shift of –2 mV is functionally similar to an increase in maximum K conductance with 55%, with no shift of the channels voltage dependence. Larger effects on the voltage dependence of the K channels are equivalent to larger increases in channel densities (Fig. 9E).

The described effects on voltage-gated K channels, mainly responsible for repolarizing the action potential, are different from that for K channels open at resting potential. KCNQ (Kv7) channels are activated at subthreshold membrane voltages and an open ion channel of this type hyperpolarizes the cell making it more difficult to elicit an action potential [261]. In contrast, more K channels of delayed-rectifier type, which are closed at a normal resting

potential, do not affect a single action potential. Instead, they affect repetitive activity because they stay open some time after the initial action potential.

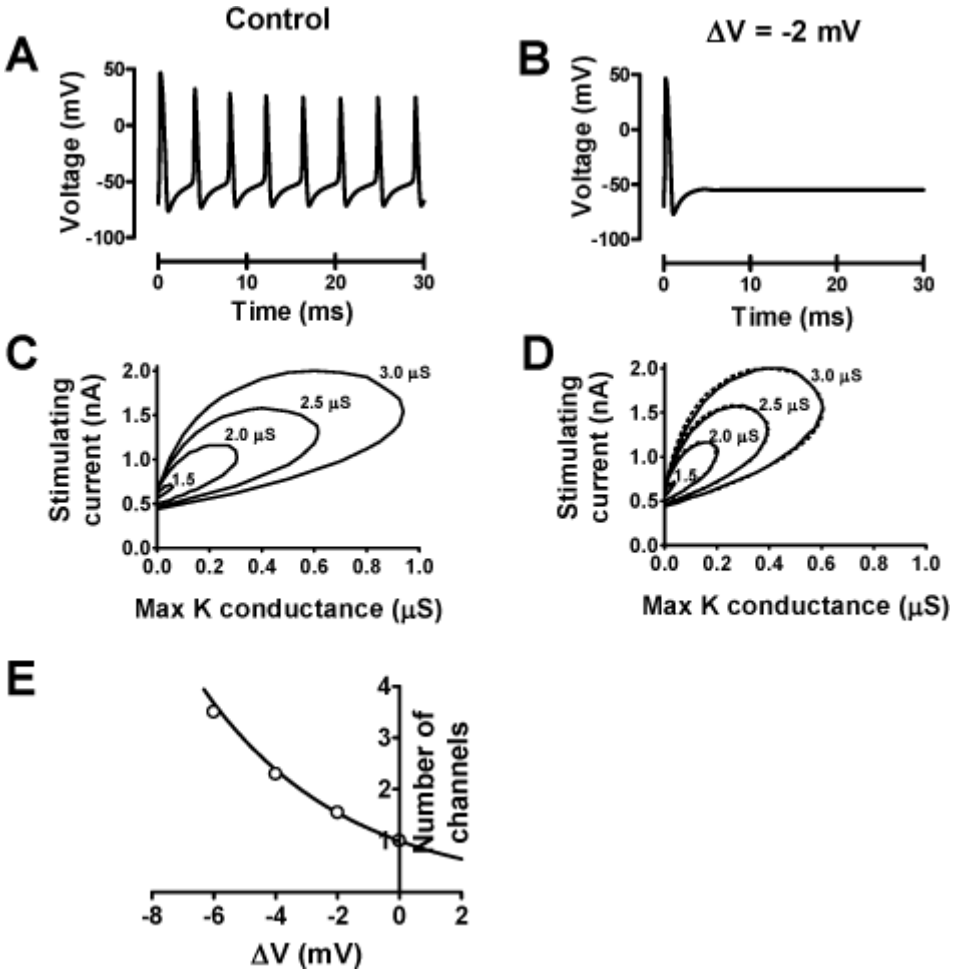

Figure 9: Small shifts of $G(V)$ have pronounced effects on excitability. (A) In computer simulations on the frog myelinated axon [260] a 0.8 nA stimulating current induces repetitive firing [128]. (B) Repetitive firing is completely abolished by shifting $G_K(V)$ –2 mV. (C) Because the relative densities of channels may vary in different preparation, we simulated the effect of different combinations of Na and K channel densities (conductances). Here, shown as a 2D-plot for the dependence of the ability to fire repetitively on maximum G_K (x-axis), stimulating current (y-axis), and maximum G_{Na} (figures associated with the four different oval areas). If the stimulating current is within the ovals for a specific combination of the G_K and G_{Na} then repetitive firing occurs. A higher G_{Na} increases the area of the oval. (D) Corresponding ovals when $G_K(V)$ is shifted –2 mV. The ovals are contracted to lower G_K by a factor of about 1.55 (dashed lines). Thus, a shift of –2 mV is functionally similar to an increase of the maximum G_K with 55% with no shift of $G_K(V)$. (E) Simulations with larger shifts of $G_K(V)$ than –2 mV show that the ovals are contracted even more with no other differences in the shape of the ovals. The continuous line is $A = \exp(-\Delta V/s)$, where $s = 4.6$ mV.

Concluding remark

Recent structural data of a voltage-gated K channel in an activated state and functional data from several investigations suggests a helical-screw motion for S4 to open an ion channel. Several substances can directly or indirectly affect this voltage-sensor movement and we anticipate that the voltage sensor will be a target for new medical drugs.

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