

Structure and Function of Ion Channels: A Hole in Four?

Voltage dependent ion channels are integral membrane proteins that contain four subunits or domains, which are thought to surround a central ion conducting pore. These subunits or domains consist of six transmembrane segments (S1-S6) and a hairpin loop between S5 and S6, which is thought to form the pore. In voltage dependent ion channels, the stochastic opening and closing behavior is controlled by voltage sensors. The molecular mechanism of how ion channels open and close as well as the structural basis for coupling to the voltage sensors are not understood. In the present paper, a new view of the channel is presented. The emphasis is shifted from the channel as a whole towards the subunits, which are proposed to function more independently than previously assumed. Based upon the observation of equidistant subconductance states in a voltage dependent K^+ channel, it is suggested that each subunit can make an equal and independent contribution to the conductance of the channel. It is furthermore suggested that channel opening and movement of the sensors are not strictly coupled: movement of the sensor is necessary for channel opening, but not sufficient. After the sensor of a subunit has moved, it is still in the "closed" conformation. Finally, a symmetry principle is introduced where only channel conformations in which all subunits are in the same "permeability¹" state, are energetically stable. Heteromeric channel conformations, that correspond to the subconductance state, are unstable and therefore short lived.

Key Words: *protein structure, permeation, subconductances, gating, patch clamp*

INTRODUCTION

Ion channels are integral membrane proteins found in every cell, where they perform a variety of important functions. As a class,

they form the molecular basis of processes like electrical and chemical signaling, excitability, adaptation, sensory transduction, learning and memory. It is therefore important to understand in detail the relationship between their molecular structure and the way they operate. Despite their variety, ion channels have one property in common that distinguishes them from other integral membrane proteins like pumps or transporters: they form small, very selective pores in the cell membrane through which certain ions can permeate, following their electrochemical gradient. The exact nature of this permeation process is presently unknown. Ion channels are unique among all proteins, because the behavior of an individual molecule can be studied using the patch clamp technique [1-3]. This makes them very suitable as a model system for structure-function studies of membrane proteins. When currents through a single ion channel are recorded using the patch clamp technique, it seems that the channel switches stochastically between two current levels, that correspond to the open (permeating) and closed (non-permeating) conformation. The amount of ions that can permeate through a channel per unit time is directly proportional to the relative amount of time the channel spends in the open state. For most channels this probability of being in the open state is being regulated by some physiological relevant factor. There are three major classes of ion channels that can be distinguished on the basis of what regulates their probability of being open: (1) membrane potential [4-6], (2) ligands [7-9], (3) GTP-binding proteins [10,11], or a combination [12,13]. Voltage dependent channels can be further subdivided according to the ion species they permeate: K^+ , Na^+ and Ca^{2+} channels. This paper will focus primarily on voltage dependent K^+ channels, but the conclusions obtained are relevant for all ion channels.

The fundamental question addressed in this paper is: how do ion channels open and close? A completely satisfactory answer to this question cannot be given, until the structure of ion channels at atomic resolution is known, but the model proposed in this paper does suggest a new search direction.

STRUCTURE? . . . FUNCTION!

Functionally, ion channels have been extensively studied. A variety of tools can be employed to investigate different aspects of ion channel function. Macroscopic currents can be recorded from an ensemble of ion channels, yielding kinetic information. The stochastic behavior of individual channels can be studied using the patch clamp technique, which can yield unique types of detailed information, not otherwise obtainable. Voltage dependent channels contain a charged voltage sensor, that moves in the membrane electric field in response to a change in membrane potential. This charge movement produces a very small electric current, the so-called "gating current", that can be recorded and analyzed. All these different types of measurements have yielded a wealth of information on the behavior of the different ion channels. In particular for voltage dependent ion channels, the amount of available data is so overwhelming that any attempt to summarize is bound to be incomplete. Therefore, I will focus on a small number of papers that are relevant for the present discussion and refer to a few excellent reviews [14,15].

Structurally, the situation is much less encouraging for ion channels. Crystallization of membrane proteins has proven to be very difficult, and has not been successful for ion channels yet. Therefore, there is at this time virtually no information on the three dimensional structure of these proteins. With the cloning of a large variety of both voltage dependent and ligand-gated ion channels, many amino acid sequences have become available. A hydrophobicity analysis of these sequences has been used to predict which parts of the protein are in the membrane (Figure 1 a and b). Based on this kind of analysis, a structural model has been proposed for voltage-dependent channels, that consists of four subunits (K^+ channels) or domains ($Na^+ Ca^{2+}$ channels), which surround a central pore [16,17]. Each subunit or domain consists of six transmembrane segments, labeled S1 through S6 (Figure 1 c and d). A small hydrophobic region between S5 and S6 is now thought to be partially in the membrane, possibly forming a beta

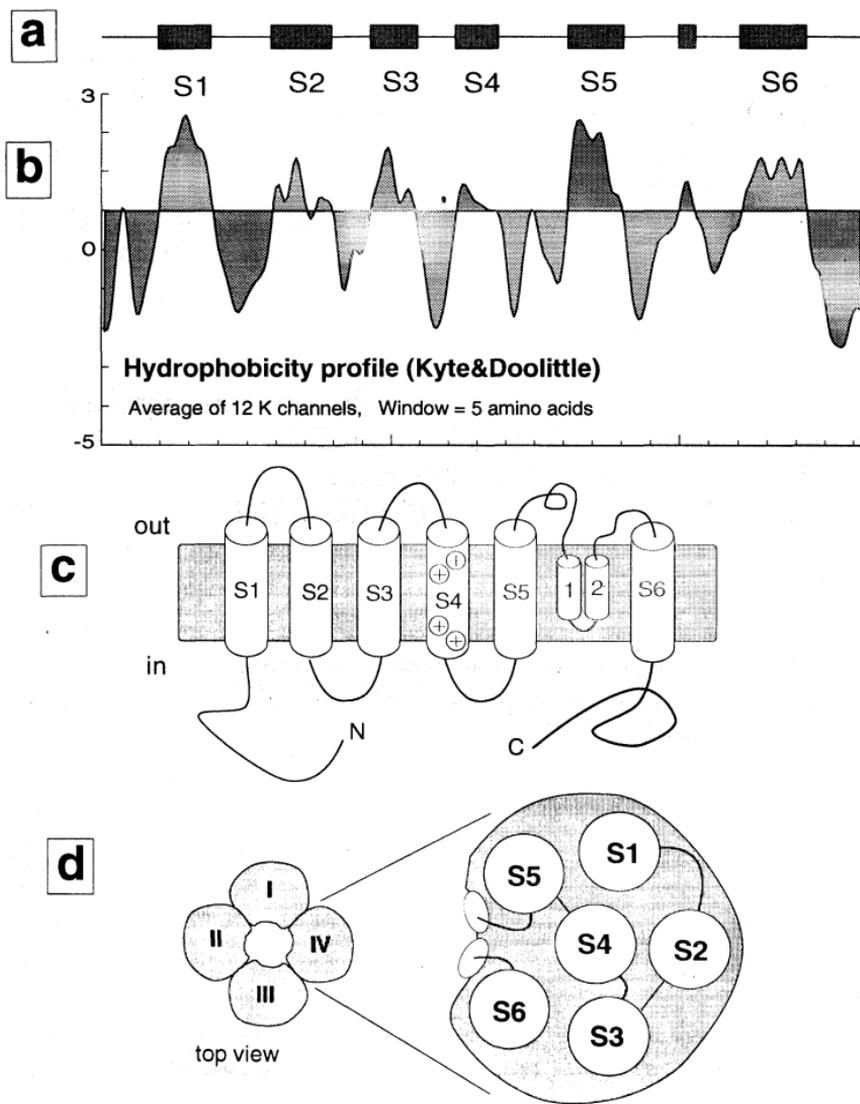


FIGURE 1 Structural model of voltage dependent ion channels. The linear amino acid sequence of voltage dependent K channels (panel a) contains 6 regions with relatively high hydrophobicity, labeled S1-S6. Panel b shows the average hydrophobicity profile of 12 K channels (*drk1*, *ngk2*, *rck1-5*, *Shaker*, *Shaw*, *Shal*, *Shab*, *Kvl*) after their amino acid sequences have been aligned. Hydrophobicity values were assigned to each amino acid using the scale of Kyte and Doolittle [see reference 30]. The 12 hydrophobicity values at each position in the alignment were averaged. Further noise reduction was obtained using a moving average with a window width of five residues. Regions with an average hydrophobicity of more than 1.0 are hydrophobic; they prefer the lipid bilayer as an environment, and are therefore thought to correspond to transmembrane segments. Six hydro-

hairpin loop (see below). The structural model arrived at is both coarse and hypothetical, but it is being tested and improved continuously by site directed mutagenesis, as explained below.

Because of the wealth of functional information on voltage dependent channels, a "functional model" of the channel had been developed long before the first amino acid sequence became available. This functional model contains both structural components (i.e., voltage sensor, "gate", pore, selectivity filter), and mechanistic elements that suggest how the different structures work together to form a functional channel. Without this functional model, the amino acid sequence alone would never have yielded a structural model as detailed as the one shown in Figure 1.

STRUCTURE FROM FUNCTION

In 1952, a series of papers was published by Hodgkin and Huxley [18], of which the influence can be felt even today. They introduced the voltage clamp technique, described measurements of Na^+ and delayed rectifier K^+ currents in squid axons and carefully analyzed their voltage dependence and kinetics. When currents were activated by a depolarizing voltage step, they observed sigmoidal activation kinetics, while deactivation following a hyper-polarizing step had an exponential time course. Based on this

phobic regions each approximately 20 amino acids long, have been assigned in all voltage dependent K channels cloned so far, as well as in every domain of all voltage dependent Na and Ca channels. Since it takes about 20 amino acids for a protein to cross the membrane as an alpha helix, it has been suggested that S 1 -S6 have such a conformation. The small hydrophobic region between S5 and S6 is now thought to line the pore of the channel, as explained in the text. The assumption that the amino and carboxyl terminus are localized intracellularly then completely defines the (two dimensional) transmembrane folding pattern (panel c). The S4 segment forms the voltage sensor, because it contains several positively charged residues. Because of its amphiphilic character, S4 cannot face the bilayer, and because it needs to experience the membrane electric field it cannot face the ionic solution. For these reasons, S4 has a central position in the 3-D model shown in panel d. Since the linker region between S5 and S6 is relatively short in K channels, S5 and S6 are positioned close to the pore. This leaves S1, S2 and S3 to face the bilayer, and their relative position was assigned arbitrarily.

observation, and the steep voltage dependence of the kinetics, they proposed the following model for the activation of the delayed rectifier K^+ channel. They suggest that the voltage dependence results from charged particles, which are localized within the membrane electric field, where they sense the membrane potential. At negative membrane potentials, these voltage sensors would be in a resting position, preventing the channels to open. At more depolarized potentials the particles would move to an active position, in a first order reaction. The transition rate constants were assumed to be an exponential function of membrane potential. To explain the sigmoidal activation kinetics of the delayed rectifier K^+ channel, they suggest that there are four of these particles per channel, that they are independent, and that they all four need to move before the channel can open (Figure 2a). They also note that the movement of these charged particles across the membrane should result in a small electric displacement current, which they fail to measure. Much later these predicted displacement currents, which are usually referred to as "gating currents", were experimentally verified in the squid axon [19] and at present they can be studied with high resolution, using cloned ion channels [20,21]. These experimental observations strengthened the idea that ion channel proteins should contain a charged structural element, that is being moved by the electric field preceding activation of the channel.

Early experiments were all done using macroscopic ion currents. Although the ensemble average of the behavior of a large number of channels is deterministic, it was realized that the behavior of the individual channels had to be stochastic. In an influential paper published in 1968, Verveen and Derksen [22] showed that noise present in the macroscopic ion currents actually can be used as a source of information. By assuming that ion channels were stochastically switching between two conformations, "open" and 'closed", and that the open channel had a conductance that was constant and specific (the "unitary conductance" hypothesis), it was possible to estimate both the single channel conductance and the mean open time from the variance in the mean ion current [23].

This "unitary conductance" hypothesis used in all noise experiments seemed to be confirmed when the behavior of individual channels was experimentally observed using the patch clamp technique [2,24]. The resolution of these single channel recordings was dramatically improved by the giga-seal technique, and the development of low noise patch clamp amplifiers [1]. The structural implication would be that the ion channel protein must have at least two different conformations, corresponding to the open and closed state (Figure 2 b), between which it alternates stochastically.

The actual mechanism by which ions move through the channel has proven to be very elusive. Since the permeation process is passive, driven by the electro-chemical gradient instead of metabolic energy, aqueous diffusion is a logical first model to consider. In this case the pore is a water filled hole through the channel. The Goldman-Hodgkin-Katz permeability equations [4] are very popular for describing the non-linearity which is often seen in the current-voltage relationship of an open channel in non-symmetrical ionic conditions. These equations are in fact derived for aqueous diffusion in a long narrow cylinder. A major problem with this diffusion model of the channel pore is that it cannot explain the ion selectivity observed in these channels. For this reason alternative models have been put forward [25-27]. Channels are thought to contain a "selectivity filter", a narrow portion of the channel, that is able to partially dehydrate the ions and interact closely with them. In "barrier hopping" models, the ions are thought to pass through a series of potential energy barriers and wells, when it interacts with the protein while it crosses the membrane. Selectivity is brought about because the energy profile, the height and width of the barriers and wells, depends on the ion species. Structurally, this implies that there are low affinity ion binding sites within the pore (wells) and hydrophobic or narrow regions where ions do not like to go (barriers). When the number of barriers and wells becomes very large, a situation similar to diffusion arises, where the pore lining replaces the aqueous environment [28]. It is presently not clear whether selectivity arises from differences in the barriers, the wells, or a combination.

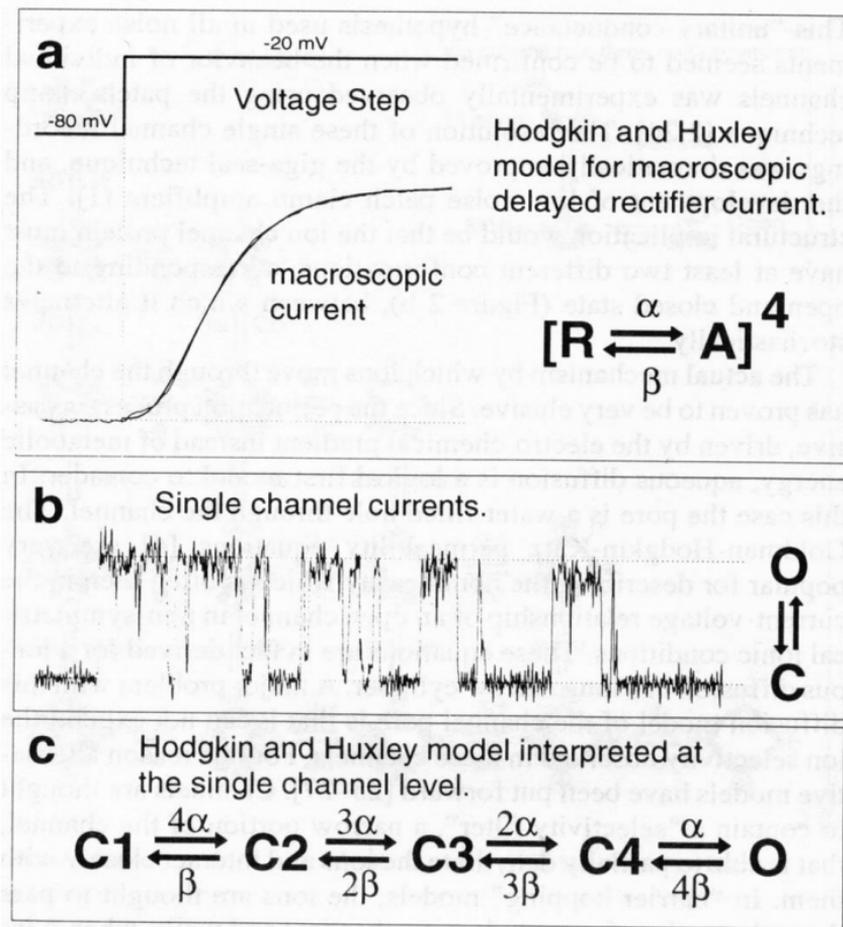


FIGURE 2. Functional models of voltage dependent channels. Delayed rectifier K channels display sigmoidal activation kinetics. This is illustrated here for a K channel from rat brain, *drkl* [30]. The current activated by a 100 msec voltage step from -80 mV to -20 mV, clearly has a sigmoidal time course (panel a). Superimposed on the current trace is shown a fit with the Hodgkin and Huxley n^4 model for the activation of the delayed rectifier in squid axons. They propose the existence of charged particles, whose behavior is governed by the membrane electric field. These particles can be in two positions, which I have called resting (R) and active (A). The forward and backward rate constants α and β are a function of membrane potential. Negative membrane potentials favor the resting position, while depolarized potentials favor the active position. When the membrane potential is suddenly altered from -80 mV, where all the channels are in R, to -20 mV, the number of particles in the active position, $n(t)$, changes with the following, mono-exponential, time course:

$$n(t) = n_{inf} (1 - \exp(-t/\tau))$$

THE STRUCTURE-FUNCTION MODEL

The ideas in the previous section have resulted in a structure-function model of ion channels, even before any structural data were available (Figure 3). The model proposed by Hodgkin and Huxley to explain the activation kinetics of the macroscopic current has been interpreted at the single channel level, by assuming that the channel is closed, unless all four charged voltage sensors are in the active position [29]. This implies a strict structural coupling between the voltage sensors and the open/close mechanism.

A second consequence of over-interpreting Hodgkin and Huxley's model is that the channel open/close mechanism is often thought as being mechanical in nature: since it is the *movement* of the sensors that causes the channel to open, it is assumed that the actual opening of the channel also involves the movement of a structural element, usually referred to as the "gate". This gate is thought to obstruct the pore in the closed conformation, moving out of the way when the channel opens. In this view of the channel, the part of the pore that determines permeability and selectivity is static, all the open/close dynamics are situated in the mechanical gate. The idea of a static permeability structure is also consistent with the unitary conductance hypothesis.

This paper is going to challenge certain aspects of this structure-function model. The experiments described here were performed

where n_{inf} is the steady state value of the number of particles in the active position and τ is the activation time constant. To explain the sigmoidal nature of the activation kinetics, Hodgkin and Huxley state: "*potassium ions can only cross the membrane when four similar particles occupy a certain region of the membrane*" [18]. Therefore, the macroscopic current I_k is proportional to $n(t)$ raised to the fourth power: $I_k = g n^4$, where g is a scaling factor. Panel b shows a 400 msec single channel current trace recorded from *drk1* at 0 mV. The channel can be seen to switch stochastically between what seems to be two current levels, open and closed. The simplest way to link the macroscopic description (a) with the behavior of an individual channel (b) is to equate the conformation where all four particles are in the active position with the open state of the channel (panel c). The result is that the macroscopic time constant of activation and the open and closed times at the single channel level become linked, since they all depend on the forward ($n\alpha$) and backward ($n\beta$) rate constants, where $n = 1, 2, 3$ or 4 as indicated.

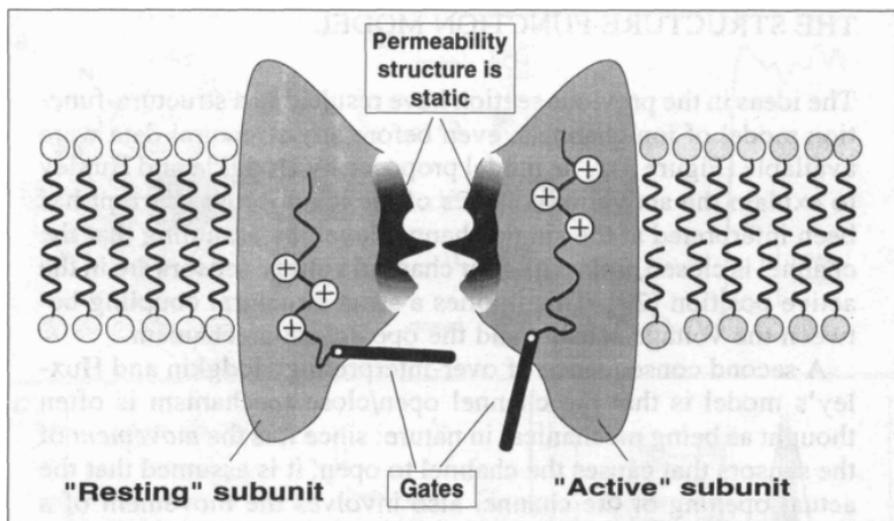


FIGURE 3 Classical structure-function model of voltage dependent ion channels. Voltage dependent ion channels are integral membrane proteins that form ion selective pores. They are thought to have two wide vestibules and a narrow central pore region that determines permeation properties. This permeability structure is usually thought of as being static, not involved in the opening and closing dynamics of the channel. The channel consists of subunits and every subunit contains a charged structure (S4 in Figure 1) that senses the membrane voltage. These voltage sensors are suggested to be mechanically linked to the "gates" that are responsible for opening and closing the channel. These gates close the channel by physically obstructing the pore. Movement of the voltage sensor is directly coupled to movement of the corresponding gate.

using *drk1*, a delayed rectifier K^+ channel cloned from rat brain [30]. Based on results obtained using point mutations, and the analysis of subconductance levels in this K^+ channel, the following model is proposed: (1) movement of the voltage sensor is necessary but not sufficient to open the channel, (2) the subunits/domains that constitute a channel make independent contributions to permeation, (3) only channel conformations in which all the subunits have the same permeability state are energetically stable, and (4) the permeability structure is dynamic, and forms the structural basis for the open/close mechanism.

MUTAGENESIS: IMPROVING THE STRUCTURE-FUNCTION MODEL

Three things have contributed to the proposed folding pattern of the ion channel protein, which is illustrated in Figure 1: (1) the functional model of the channel described above, (2) the hydrophobicity analysis of amino acid sequences, and (3) site-directed mutagenesis, which will be discussed next. The amino acid sequence of every voltage dependent channel cloned so far shows a conserved motif of a core region containing six putative transmembrane segments (S1-S6), flanked by hydrophobic domains. In voltage dependent K^+ channels these hydrophobic domains correspond to the amino (N) and carboxyl (C) terminus (Figure 4). To find out what the functional role of the N- and C-terminus is, a consecutive series of deletions in the termini was made in *drk1*, and the mutants were expressed in *Xenopus* oocytes (Figure 4). It was found that the deletion mutants that removed part of the S1-S6 core region did not express functional channels in the oocyte. Mutants that left the core region intact were functional, although their kinetics of activation as well as their inactivation properties was often altered [31]. The results also suggested that the C- and N-terminus interact, which is consistent with them being on the same side of the membrane. These findings strengthen the proposed folding model, and focus the attention on the S1-S6 core region.

Immediately following cloning of the first voltage dependent ion channel [32], it was suggested that the fourth transmembrane segment (S4) constitutes the voltage sensor, because it contains a striking pattern consisting of a series of positively charged amino acids, separated by two hydrophobic residues [16]. Since then, several papers reporting site-directed mutagenesis of both the charged and hydrophobic amino acids have confirmed the importance of S4 for voltage sensing [33-35].

A small hydrophobic region between S5 and S6 was predicted to be partially in the membrane and because it was highly conserved it was suggested that it forms the pore of the channel [36]. This prediction was later confirmed by three groups using site-directed mutagenesis and chimeric channels [37-39]. It was sug-

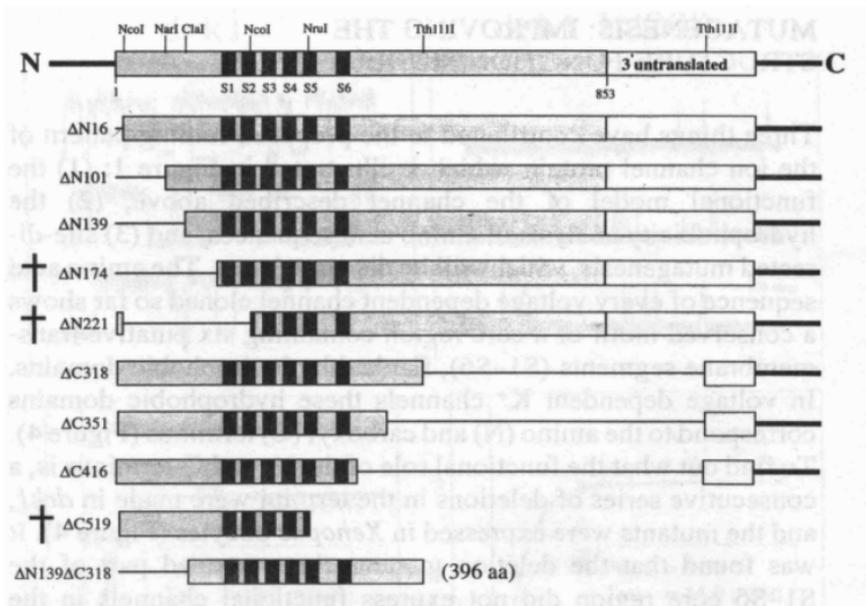


FIGURE 4 Deletions at the N- and C-terminus of *drk1* delineate the transmembrane region. *Drk1* is a delayed rectifier K channel cloned from rat brain. The protein is 853 amino acids long and consists of a putative core region (S1 -S6), a relative short N-terminus and a long C-terminus. The top of the figure shows the *drk1* cDNA clone, where the coding region is shaded, the putative transmembrane regions are shown in black and a few relevant restriction sites are indicated. A progressive series of deletion mutants was constructed and expressed in *Xenopus* oocytes [31]. The number of amino acids deleted is indicated at the left of each construct. A cross to the left of a construct indicates that there was no detectable functional expression.

gested that four linker regions form a beta barrel structure [39] and molecular modeling of this region has shown that such a structure is feasible [40].

Taken together, these findings have underscored the predictive power of the hydropathy analysis, and further helped to detail the structural working hypothesis of the voltage dependent channel. In the next section some recent site-directed mutagenesis results will be discussed, that support the idea that voltage sensing and channel opening are two separate processes.

GATING AND "GATING"

After carefully analyzing the voltage dependence of the single channel behavior of the *Shaker* K^+ channel, Zagotta and Aldrich [41] introduced into the Hodgkin and Huxley model (Figure 2c) an additional, voltage independent conformational change necessary to open the channel. This suggests that sensor movement and channel opening are two separate physical processes, which has also been proposed in a recent theoretical study [42]. As a result, the term "gating" becomes ambiguous, because it refers both to the movement of the sensors (like in "gating current") and the open/close behavior of the channel (like in "fast and slow gating" [43]). New experimental evidence confirms that movement of the sensor has a structural basis which is different from opening/closing of the channel. In voltage dependent K^+ channels, the fifth transmembrane segment (S5) is flanked on either side by a strictly conserved glutamate residue. This negatively charged amino acid is unique for K^+ channels: it is not present at the corresponding position in Na^+ or Ca^{2+} channels. A point mutation was introduced in *drk1*, substituting the glutamate on the N-terminal (cytoplasmic) side of S5 to an aspartate, thereby retaining the charge, but changing the bulkiness of the side chain. This very conservative mutation seemed to have little effect on the macroscopic activation kinetics, but had a dramatic effect on the single channel behavior [44]. The mean open time, which is around 20 msec in *drk1*, was reduced more than 10-fold in the mutant to less than 2 msec. Closed time intervals were prolonged in the mutant. In contrast, activation kinetics were not affected: the time constant of activation was approximately 15 msec at 0 mV in both cases (Figure 5). If movement of the sensor would be strictly coupled to opening of the channel, than a substantial change in single channel behavior should coincide with a change in activation kinetics, which was not observed. It is therefore suggested that movement of the voltage sensor is necessary to open the channel, but it is not sufficient (Figure 6). In addition it suggests a key role for this conserved glutamate in stabilizing the open state of the channel. The opening mechanism itself will be considered next.

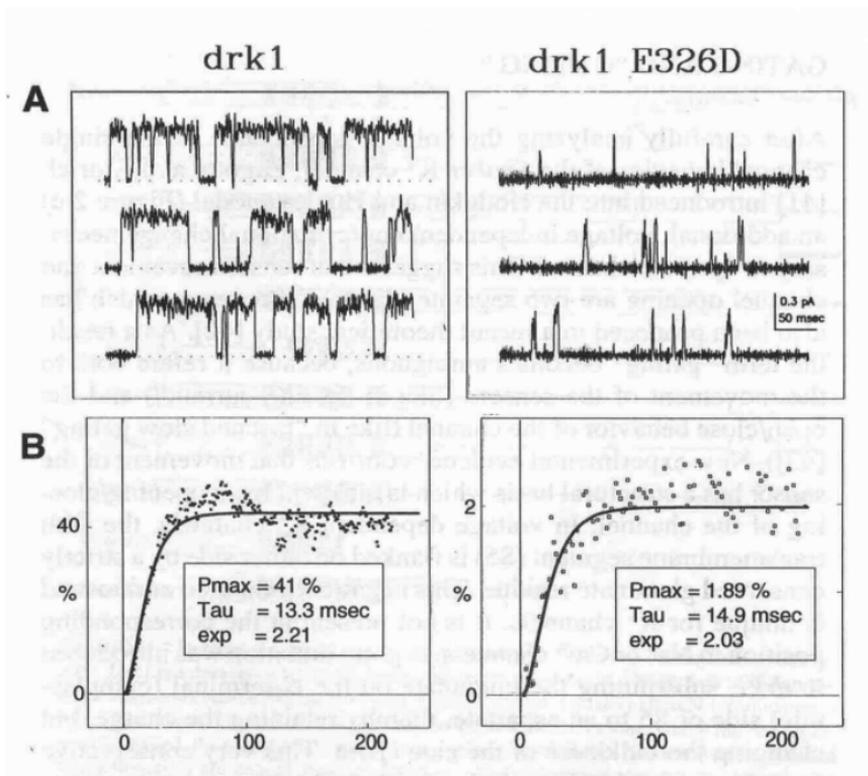


FIGURE 5 A conserved glutamate is involved in stabilizing the open state. In voltage dependent K channels, but not in Ca and Na channels, the fifth transmembrane segment (S5) is flanked on either side by a strictly conserved glutamate residue. This amino acid was mutated to aspartate in *drk1*, thereby retaining the negative charge while changing the bulkiness. This very conservative mutation dramatically affected the single channel behavior without having a large effect on the macroscopic current. Panel A shows three single channel current records for both *drk1* and the mutant E326D. This activity was elicited by 400 msec pulses to 0 mV, from a holding potential of -100 mV. The mean open time in *drk1* is approximately 20 msec, but is reduced at least 10-fold in the mutant channel. Panel B shows the ensemble average of several hundred single channel records (dots), fitted with the Hodgkin and Huxley n^3 model:

$$P(t) = P_{\max} * [1 - e^{-t/\tau}]^a$$

where $P(t)$ is the probability of being in the open state as a function of time. Three parameters are optimized: P_{\max} (the steady state open probability), τ (the activation time constant), and a (the exponential). The mutation did not significantly affect the time constant of activation, but only reduced the steady state open probability.

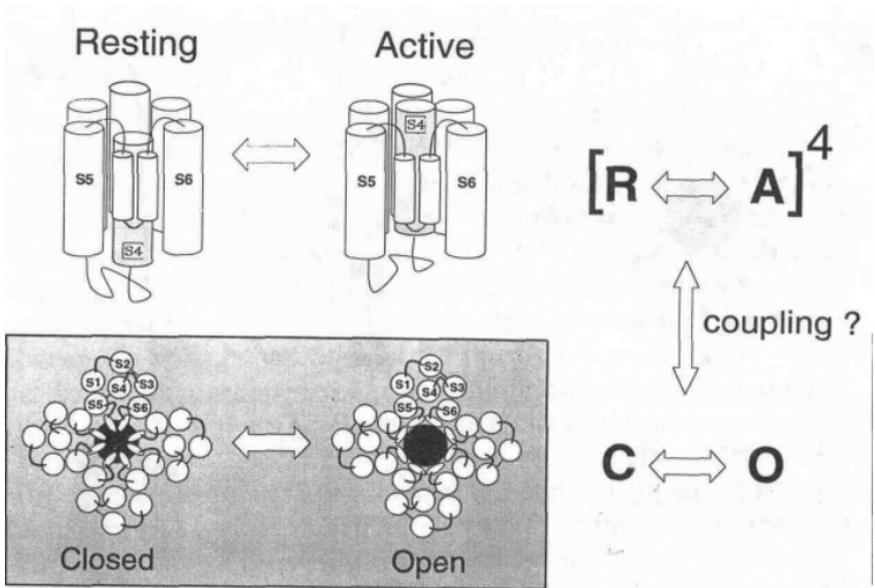


FIGURE 6 Activation vs. Opening. As shown in the previous figure and explained in the text, it is possible to dramatically alter single channel open/close behavior without affecting activation kinetics. This suggests that activation of the channel is a process that can be distinguished from the actual opening of the channel. Although activation is required for channels to open, it is not sufficient. Activation involves movement of the charged voltage sensors (S4 segments) from the resting to the active position. Therefore, every subunit can be in a resting or active conformation. It is usually assumed that the channel can only open after all four sensors have moved. Opening and closing of the channel is a distinct physical process that probably involves a conformational change at the level of the pore. The two processes are coupled by a yet unknown mechanism.

SUBUNITS AND SUBCONDUCTANCES: A HOLE IN FOUR?

Voltage dependent K^+ channels are thought to assemble from four identical subunits, each with its own voltage sensor (S4) and each making its own contribution to the formation of the pore. In the current structural model, the subunits surround a central pore, therefore K^+ channels are four-fold symmetrical (Figure 7a). What is the role of the subunits in voltage sensing, channel opening/closing, and ion permeation? Since the four subunits that form a K^+ channel are identical, the subunits must have a conformation

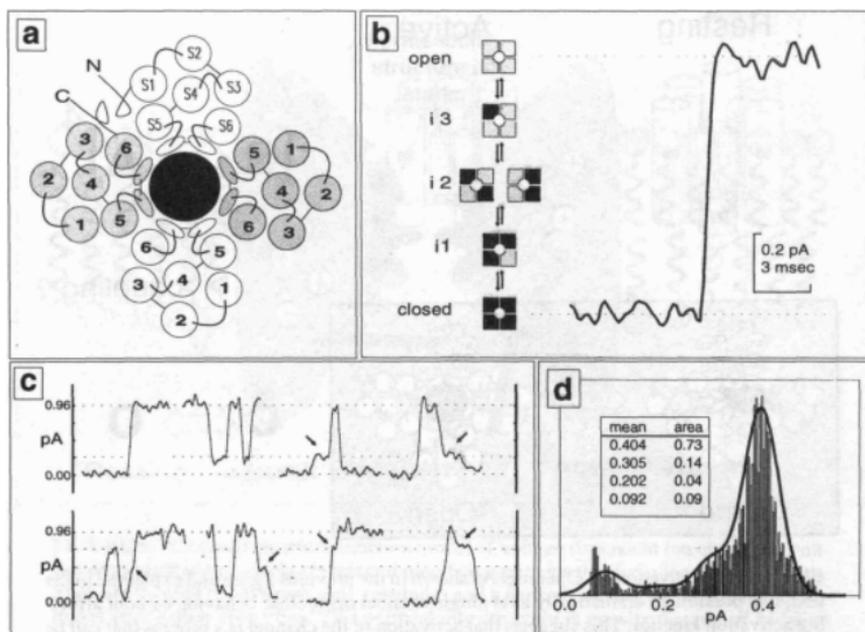


FIGURE 7 Subunits and subconductances. Voltage dependent K channels consist of four identical subunits that surround a central pore. Panel a shows a top view of the channel containing four subunits, each with 6 transmembrane segments. The S5-S6 linker region is shown to line the pore. Irrespective of the actual mechanism of channel opening, every subunit will have two conformations that correspond to the open and closed state of the channel. Unless conformational changes in the four subunits occur simultaneously, there should be intermediate, heteromeric channel states, where some subunits are in the open conformation and others are closed (panel b). Whenever there is an opening transition, the channel has to visit these intermediate states successively. When opening and closing transitions of *drk1* were studied carefully, it was found that there were many instances where the channel seemed to pause briefly at intermediate current levels, giving the appearance of shoulders to the transitions (arrows in panel c). The amplitudes of these short lived sub-conductance levels were not continuously distributed, as would be expected if they were filter artifacts. Instead, they fell into three classes with amplitudes of approximately 25%, 50% and 75% of the full conductance level. Panel d shows a single channel amplitude histogram that was fitted with a sum of 1 to 5 Gaussians. Four Gaussians were necessary and sufficient to describe the amplitude distribution. It is therefore suggested that these three short-lived equidistant subconductance states that occur mainly at transitions, correspond to the three heteromeric intermediate states i1, i2, and i3 in panel a.

that corresponds to the open and closed state of the channel. It is unlikely that conformational changes in the subunits occur simultaneously. This implies that there should be heteromeric states, in which some of the subunits are in the open conformation, and others are closed (Figure 7b). Whenever there is an opening transition, the channel has to visit these intermediate, heteromeric states. According to the unitary conductance hypothesis these intermediate states are not permeating ions, and therefore indistinguishable from the homomeric closed state.

Looking superficially at single channel recordings, like the data shown in Figure 5 a, seems to confirm this idea. However, when the same data is studied carefully at high resolution, a different picture arises. Although many opening transitions seem to go directly from the closed state to the open state (Figure 7 b and first transition of 7 c), there are many instances where the channel seems to pause briefly at an intermediate current level, giving the appearance of shoulders to the transitions (arrows in Figure 7 c). When the amplitude distribution of these short lived levels was studied, it was found that there are three subconductance levels, with amplitudes approximately equal to 1/4, 2/4 and 3/4 of the fully open channel (figure 7 d). A complete analysis of this data is the subject of a full length paper (VanDongen and Brown, in preparation). These short lived subconductance states correlated strongly with opening and closing transitions. Because of these properties, it is suggested that the subconductance states correspond to the putative heteromeric states shown in Figure 7 b. This implies that the individual subunits make an equal and independent contribution to the total permeability of the channel [45,46]. The fact that subconductance states are short lived might be related to the asymmetric nature of the heteromeric conformations, which could be energetically unstable. The homomeric fully open and fully closed state are symmetrical, and therefore long lived.

The above ideas are summarized in the model shown on Figure 8. The model focuses on the individual subunits. It assumes that a subunit can be in one of three conformations, which are called resting, active and open. Subunits are not contributing to permeation, when they are in either the resting or active conformation. The difference between these conformations is the position of the

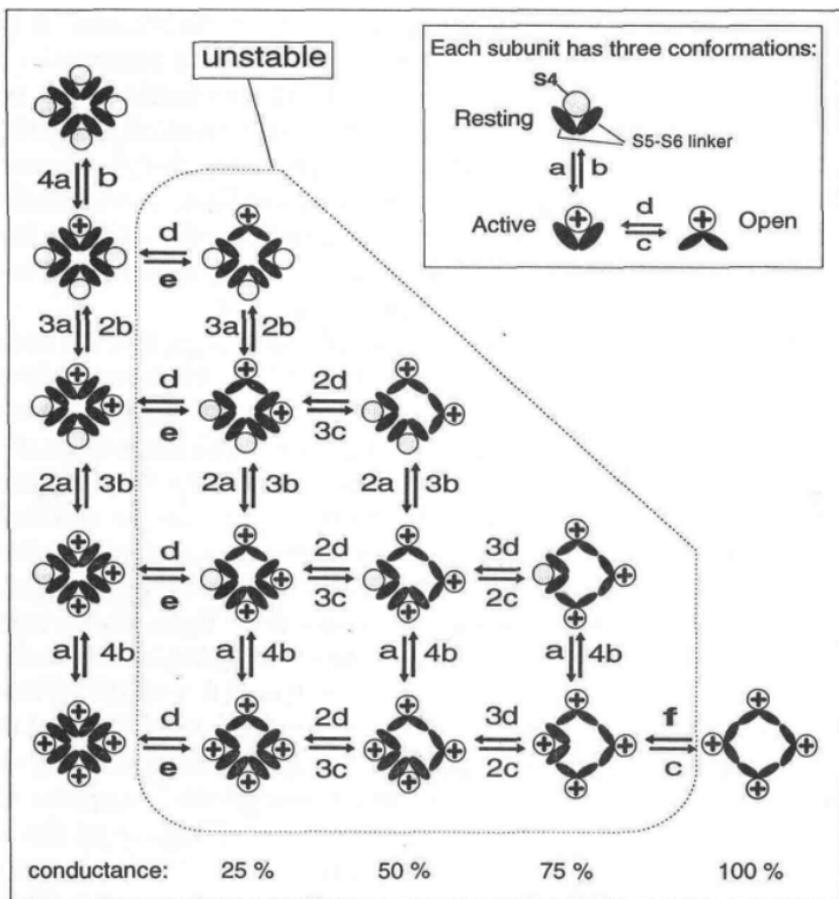


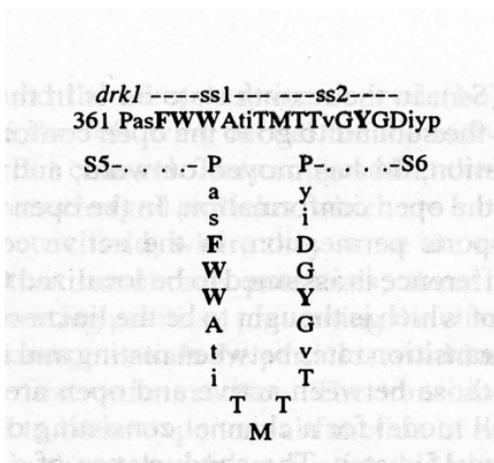
FIGURE 8 The subunit-subconductance model. Two ideas were developed: (1) activation is necessary but not sufficient for channel opening, and (2) subunits make an independent and equal contribution to permeation. These two concepts are now integrated in a new structure-function model. Activation and channel opening are explicitly modeled as being separate processes. The voltage sensor S4 can be in two positions, resting or active. The S5-S6 linker region has two conformations, one that supports permeation and another one that does not. Since activation is required for channel opening, each subunit can be in one of three conformations: resting, active or open. Permeation is supported only in the open conformation. In the resting conformation the voltage sensor (S4) is in the inward position. Activation involves outward movement of the charged sensor and brings the subunit in the active conformation. A subunit cannot go directly from the resting to the open conformation: activation is necessary for opening. The rate constants **a** and **b** are voltage dependent, and are equivalent to α and β in Figure 2a. The transition from active to open does not involve translocation of charges across the membrane electric field. Therefore, rate constants **c** and **d** are voltage independent. This linear 3-state model of the subunit is

voltage sensor (S4): in the resting state S4 is in the inward position, preventing the subunit to go to the open conformation. In the active conformation, S4 has moved outward, and the subunit is allowed to visit the open conformation. In the open conformation, the subunit supports permeation, in the active conformation it does not. The difference is assumed to be localized to the pore, the structural basis of which is thought to be the linker region between S5 and S6. The transition rates between resting and active are voltage dependent, those between active and open are voltage independent. The full model for a channel consisting of four of these subunits contains 15 states. The conductance of a state is determined by the number of subunits in the open conformation, which increases from left to right in Figure 8. States which have asymmetric pores, with some subunits supporting permeation and others not, are unstable and therefore short lived.

STRUCTURAL IMPLICATIONS

The proposed symmetry principle has important structural implications, because it points towards the existence of ring structures at the level of the pore, that are stable yet dynamic. The S5-S6 linker region that is thought to form the pore is bounded by two prolines, and consists of a loop with two limbs, usually called *ssl* and *ss2*. This region contains several conserved aromatic residues that might play a role in forming the ring structures that are involved in opening and closing. These ring structures stabilize through subunit-subunit interactions at the level of the pore. In the (8-barrel models investigated by Bogusz and Busath [40], the pore is formed by 4 pairs of anti-parallel β -strands, in which *ssl* of one

expanded to the 15-state model of the channel as shown in the figure, by making two additional assumptions: (1) subunits are independent, and (2) states with homomeric pores are stable, heteromeric states are unstable. This last assumption can be seen as an exception to the first one. It results in an additional two rate constants *e* and *f*, which are small relative to *c* and *d*. Therefore, there are only six independent rate constants in the complete model. The conductance of the channel is proportional to the number of subunits that support permeation.



S5-S6 linker region of *drk1*.

The putative pore-forming region is bounded by two prolines. It consists of two limbs: *ssl* and *ss2*. Variable residues are shown in lower case. Conserved aromatic residues are bolded.

subunit is hydrogen bonded to *ss2* of the neighboring subunit. Bulky aromatic side chains pointing into the pore are forming a narrow constriction, that is suggested to be involved in ion selectivity. The tyrosine (Y) in the strictly conserved GYGD sequence is particularly interesting in this respect, since it is flanked by two glycines that provide a large amount of flexibility.

This tyrosine plays a key role in an interesting model proposed by Lee [47]. In this model the four tyrosines are pointing into the pore, forming a ring when their hydroxyl groups hydrogen bond to each other. The diameter of this ring would be too small for K^+ ions to pass, and therefore the channel would be closed in this conformation. In Lee's model, the channel opens when this ring structure is destabilized by electron transfer from the tyrosine to one of the tryptophanes, followed by de-protonation of the tyrosines. This results in formation of a ring of oxygen radicals that would facilitate dehydration of K^+ ions and be wide enough for partially dehydrated K^+ ions to pass through. This model is important for several reasons. It is the first model to explicitly define the closed and open conformation of the channel at the atomic level. Second, it proposes for the first time a detailed physical mechanism for switching between the two conformations. And finally it illus-

trates the importance of symmetry for stability. We have now arrived at a view of the pore that is more dynamic than previously assumed. The difference between the closed (active) and open conformation of a subunit could be a subtle difference in hydrophobicity or charge density somewhere in the channel lining. Rather than having a physical structure, or "gate" moving in and out of the pore, it is conceivable that the permeability structure itself is changing its properties, when the channel opens and closes.

The subunit-subconductance model proposed above states that an individual subunit can support ion permeation and that every subunit makes an equal contribution to the total conductance of the channel. There are several mechanisms that one could propose to explain this phenomenon. A straightforward one would be a multi-barrel channel, where every subunit forms a pore. These four pores would need to be in intimate contact to explain the stabilization of the homomeric conformations. Since a multi-barrel channel is not in line with the current structural working hypothesis (Figure 1), it is important to look for mechanisms based on a single pore. Such a pore would be four-fold symmetric, with a wall consisting of four identical parts. Every subunit makes an equal contribution to the energy barriers and wells that an ion encounters when it moves through the pore. The rate with which a particular ion permeates is determined by one or more energy wells or barriers. If the channel opens and closes by changing the properties of these rate-determining wells and barriers, it is easy to see how the conductance would depend on the number of subunits contributing to a critical barrier or well. What is not immediately clear is why every subunit would make an *equal* contribution to the total conductance, since the relationship between barrier height and conductance is nonlinear in most models. This question has been explicitly addressed by Dani and Fox [48], who have shown that regular subconductance levels can arise in three theoretical ion permeation models, using a single aqueous pore.

The subunit-subconductance model of the channel, as illustrated in Figure 8, makes a number of predictions that can be tested experimentally. It explicitly takes into account the subunit struc-

ture of the channel, and might therefore provide an improved framework for future structure-function studies.

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