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MINI REVIEW

Using Mutagenesis to Study Potassium Channel Mechanisms

Roderick MacKinnon[1]

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Abstract

The voltage-activated K^+ channels are members of an ion channel family that includes the voltage-activated Na^+ and Ca^{2+} channels. These ion channels mediate the transmembrane ionic currents that are responsible for the electrical signals produced by cells. The recent cloning of numerous voltage-activated K^+ channels has made it possible to combine molecular-genetic and biophysical methods to study K^+ channel mechanisms. These mutagenesis-function studies are beginning to provide new information about the architecture of K^+ channel proteins and how they form a voltage-gated, K^+-selective pore.

Key Words: Potassium channels; membrane proteins; channel gating; ion permeation; mutagenesis; channel structure; shaker; TEA; charybdotoxin.

Introduction

Potassium channels play a central role in a wide variety of biological processes. Some K^+ channels are involved in cell volume regulation, some mediate hormonal secretion, and others control the shape and frequency of the electrical impulses generated and propagated by neurons and other electrically excitable cells. Potassium channels, like other ion channels, catalyze one of the simplest imaginable reactions: the passive diffusion of inorganic ions across a cell membrane. But in order to do this in a biologically useful way, ion channels must exhibit many fairly sophisticated properties. For example, a K^+ channel must be able to switch rapidly between closed and open conformations in response to an external signal. Depending on the type

[1]Department of Cellular and Molecular Physiology, Harvard Medical School, Boston, Massachusetts 02115.

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of K^+ channel, the signal may be a change in the transmembrane electrical potential, a change in the concentration of intracellular Ca^{2+}, or specific modulation by other intracellular mechanisms. Once open, the channel must then mediate an extremely high throughput rate of greater than one million ions per second. At the same time it has to be extremely selective so that only K^+ permeates and other ions are excluded. These properties of signal-controlled gating and selective ion permeation allow the different K^+ channels to fill their many roles in biology.

The identification, isolation, and functional expression of the Shaker A-type K^+ channel gene from *Drosophila* marked the beginning of a new chapter in our understanding of one class of K^+ channels, the voltage-activated K^+ channels (Tempel *et al.*, 1987; Pongs *et al.*, 1988; Kamb *et al.*, 1987). This important accomplishment immediately led to the rapid identification of numerous other closely homologous voltage-activated K^+ channel genes. It also initiated a new approach to the study of the molecular mechanisms of K^+ channels. For the first time, a K^+ channel could be studied using the powerful combination of molecular-genetic and electrophysiological methods. Molecular-genetic techniques provide a way to manipulate the channel molecule under study; the composition of subunits expressed can be controlled, and the primary amino acid sequence of the K^+ channel protein can be altered almost at will. Electrophysiological methods allow the functional consequences of such manipulations to be observed and studied at an extraordinarily high-resolution level.

The combined molecular-genetic/electrophysiological approach has already begun to provide us with information about the workings of K^+ channel molecules, and this information is beginning to shape our view of how a protein may fold up in a membrane to form a gated, K^+-selective pore. I make no attempt here to review the recent literature, as any attempt to do so would be outdated in a matter of months. Rather, I have chosen to focus on a few specific examples in order to demonstrate the utility of a combined molecular-genetic/electrophysiologic approach to the study of K^+ channel mechanisms. These examples address three questions about the voltage-activated K^+ channels: (1) What is the subunit structure of these channels? (2) What region of the K^+ channel protein actually lines the pore through which K^+ ions diffuse? (3) How does rapid, spontaneous closure—a process called fast inactivation—occur in A-type K^+ chanels? So far we have only incomplete answers to these questions. But it is impressive nonetheless when one considers that the first voltage-activated K^+ channel, the Shaker A-type channel, was cloned only three years ago.

Before focusing on the details, I would like to make a general comment about the idea of using mutagenesis to study the relationship between ion channel structure and function. How can the approach work when we have

so little real structural information about most ion channels? The answer to this question is clear. The lack of direct structural information has been at least partially counterbalanced by the wealth of indirect structural information that stems from four decades of mechanistic ion channel research (Hille, 1984). Even without a crystal structure, we know that an ion channel protein must somehow form a membrane-spanning aqueous pore for ionic diffusion, and in many cases we have a reasonably complete description of the pore properties. Potassium channels, for example, must have binding sites for scorpion toxins and for quaternary ammonium compounds which are known to specifically inhibit K^+ conduction by plugging the pore (Miller *et al.*, 1985; Armstrong, 1971; Armstrong and Hille, 1972). We even have estimates of the conduction pore length for some K^+ channels (Miller, 1982; Villarroel *et al.*, 1989). And for some channels we have evidence for a gate, a part of the protein that can actually occlude the pore and thus prevent ion conduction (Armstrong and Bezanilla, 1977). These intimate details about ion channels are based on functional measurements, but they have allowed us to imagine what an ion channel probably looks like, and have made it possible to design rational experiments using site-directed mutagenesis.

Subunit Structure: K^+ Channels Are Tetramers

K^+ Channels Are Similar to a Single Domain of a Na^+ or Ca^{2+} Channel α-Subunit

Voltage-activated K^+ channels are small in size compared to their molecular cousins, the Na^+ and Ca^{2+} channels. The deduced amino acid sequences of the K^+ channels are similar in several respects to each of the four internally homologous domains or repeats of the Na^+ and Ca^{2+} channels[1]. (See the review by Catterall, 1988). A K^+ channel and a single domain of a Na^+ or Ca^{2+} channel have similar hydropathy profiles which highlight six possible membrane-spanning stretches (S1–S6 in Fig. 1A). The fourth putative membrane-spanning stretch, S4, is very atypical because the hydrophobic sequence is interrupted by a cationic residue (arginine or lysine) at every third or fourth position. This unusual motif, thought to be a voltage sensor (Stühmer *et al.*, 1989a), is very conserved: all of the voltage-activated K^+ channels have one of these peculiar sequences and the Na^+ and Ca^{2+} channels have four (one in each domain). These similarities clearly mark the voltage-activated Na^+, Ca^{2+}, and K^+ channels as related members of a common gene

[2]The Na^+ and Ca^{2+} channels are formed from a major polypeptide called an α-subunit but they also have smaller associated subunits (Catterall, 1988). Comparison here is between the α-subunit and the K^+ channel polypeptide.

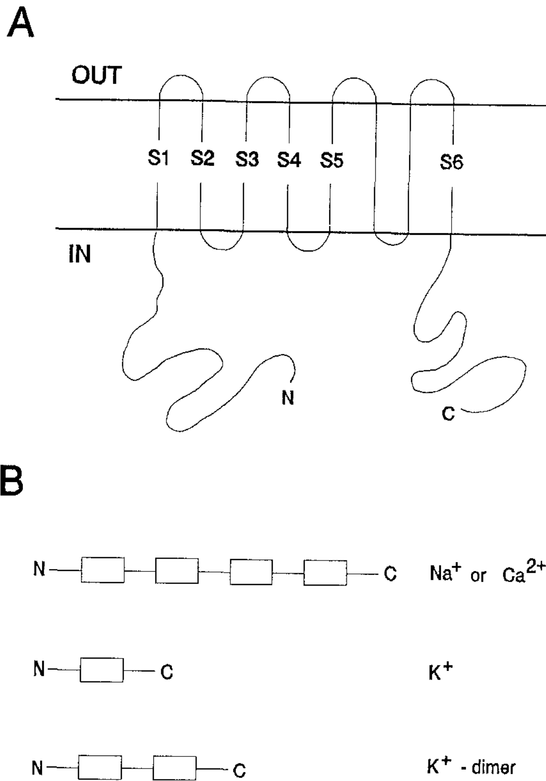

Fig. 1. (A) A hypothetical membrane-folding model for a voltage-activated K^+ channel subunit. The still uncertain topology is based on several pieces of information. Hydropathy profiles of these channels display six hydrophobic amino acid stretches which are long enough to span a membrane as an α-helix (labelled S1–S6). The amino and carboxyl termini are almost certainly located on the cytoplasmic side of the membrane (Catterall, 1988; Isacoff *et al.*, 1990; Hoshi *et al.*, 1990), and residues on the carboxyl end of S5 and amino end of S6 are known to be located on the extracellular side (MacKinnon *et al.*, 1990). The two unlabelled membrane-spanning stretches between S5 and S6 are thought to line the ion conductive pore. (B) Each box corresponds to a protein unit like the one shown in (A). Sodium channels have four linked subunits while K^+ are true tetramers.

family of voltage-activated cation channels. A distinguishing feature of the K^+ channels is their small size. The major protein unit of the Na^+ and Ca^{2+} channels look very much like four K^+ channels strung in tandem (Fig. 1B).

Hybrid Channels and Tandem Dimers

When the Shaker A-type K^+ channel gene was first cloned, its size relationship to Na^+ channels immediately suggested that a functional K^+ channel may be formed by the coassembly of independent subunits (Tempel

et al., 1987). A tetrameric structure seemed natural because each subunit would correspond to a single domain of a Na^+ channel. Evidence for multiple subunits was first provided by experiments in which two K^+ channels with distinct properties were coexpressed in the same cell (Christie *et al.*, 1990; Isacoff *et al.*, 1990; Ruppersberg *et al.*, 1990). A fraction of the resulting channels displayed a unique blend of properties: the currents could not be explained by the expression of the independent parent channels alone. For example, when a K^+ channel that is very sensitive to external TEA is coexpressed in oocytes with one that is very insensitive, the TEA sensitivity of the resulting currents is inconsistent with a sum of contributions due to both channels expressed independently (Christie *et al.*, 1990; Ruppersberg *et al.*, 1990). Similarly, when two K^+ channels with distinct gating kinetics are coexpressed, currents displaying unique, intermediate kinetics are observed (Christie *et al.*, 1990; Isacoff *et al.*, 1990; Ruppersberg *et al.*, 1990). These findings point to the formation of hybrid K^+ channels having distinct properties. Hybrid channel formation would not occur if a single K^+ channel polypeptide resulted in a functional channel, but it could if a K^+ channel is formed by the coassembly of several subunits. These results provided the first clear evidence that a functional K^+ channel is a multimeric protein.

The multimeric nature of voltage-activated K^+ channels has also been demonstrated in a completely independent way. Two K^+ channel genes were connected in a single open reading frame to form a tandem dimer (Isacoff *et al.*, 1990). Thus, a K^+ channel was designed to look like one-half of a Na^+ channel (Fig. 1B) and the construct yielded normal-appearing K^+ currents when expresed in *Xenopus* oocytes. It is possible that only one-half of the dimer is contributed to a channel complex, but more likely the result indicates that a K^+ channel is a multimer formed by the aggregation of an even number of subunits, and a single dimer fills the role of two monomers.

Counting Subunits

By coexpressing two different K^+ channels one can, in principle, count the number of subunits in a functional channel complex. The problem is a combinatorial one. Such a quantitative analysis has been carried out by studying the interaction of a scorpion toxin inhibitor, charybdotoxin (CTX), with coexpressed wild-type and toxin-resistant mutant Shaker A-type K^+ channels (MacKinnon, 1990). The mutant channel used in the study was 250 times less sensitive to toxin, but in other respects it was virtually identical to wild type. The mutant channel subunits were coexpressed along with the wild type in known ratios. By measuring inhibition of the channels at high toxin concentrations, the fraction of channels containing only mutant subunits was determined. This fraction is a measure of the subunit stoichiometry since, for a given mixture ratio, it is related in a simple binomial way to the subunit

number. For the Shaker A-type K^+ channel, the subunit number was found to be 4.

These results together have provided important information about the overall architecture of voltage-activated K^+ channels. Like other ion channels of eukaryotic cell membranes, the K^+ channels have several similar or identical subunits. Probably these subunits are arranged in a circular array to form a central ion conductive pore. Whether K^+ channels in cell membranes are homomultimers or heteromultimers has not yet been determined, and the possible role of additional minor subunits is still unknown. But we have good evidence now that the K^+ channels have four major subunits. They are tetramers, and in this respect they are very similar to the Na^+ and Ca^{2+} channels.

Identification and Characterization of Pore-Forming Residues

The gating characteristics of voltage-activated K^+ channels are quite variable, but they share a nearly invariant set of ion conduction properties. They are all highly selective for K^+ over Na^+, their pores accommodate several K^+ ions in single file, and they are inhibited by Ba^{2+}, Cs^+, and the tetraalkylammonium ions (Hille, 1984). These well-conserved functional properties suggest that the amino acid sequence and structure of the ion-conductive pore is probably highly conserved among the voltage-activated K^+ channels. Recent findings have begun to confirm this idea.

A Pore-Blocking Scorpion Toxin Identifies the Outer Mouth of the Shaker A-Type K^+ Channel

The Shaker A-type K^+ channel was found to be inhibited by the scorpion toxin CTX (MacKinnon *et al.*, 1988). A clear picture of the mechanism of CTX's action had previously emerged from studies on the interaction of the toxin with a different voltage-activated K^+ channel, a high-conductance Ca^{2+}-activated K^+ channel from mammalian skeletal muscle (MacKinnon and Miller, 1988, 1989a; Miller, 1988). Several independent lines of evidence supported a physical occlusion mechanism: the toxin prevents ion conduction by binding to and occluding the extracellular entryway to the ion-conductive pore. Furthermore, it had been shown that the highly basic toxin molecule interacts strongly, in an electrostatic manner, with negatively charged groups near the toxin binding site on susceptible K^+ channels (Anderson *et al.*, 1988; MacKinnon *et al.*, 1989).

The electrostatic interaction between CTX and a K^+ channel provided a rational basis for using the toxin as a probe in order to identify amino acid residues that line the outer mouth of the Shaker A-type K^+ channel. Point mutations involving charged residues in several regions of the protein had no effect on CTX inhibition, while mutations in the S5–S6 linker (the stretch of

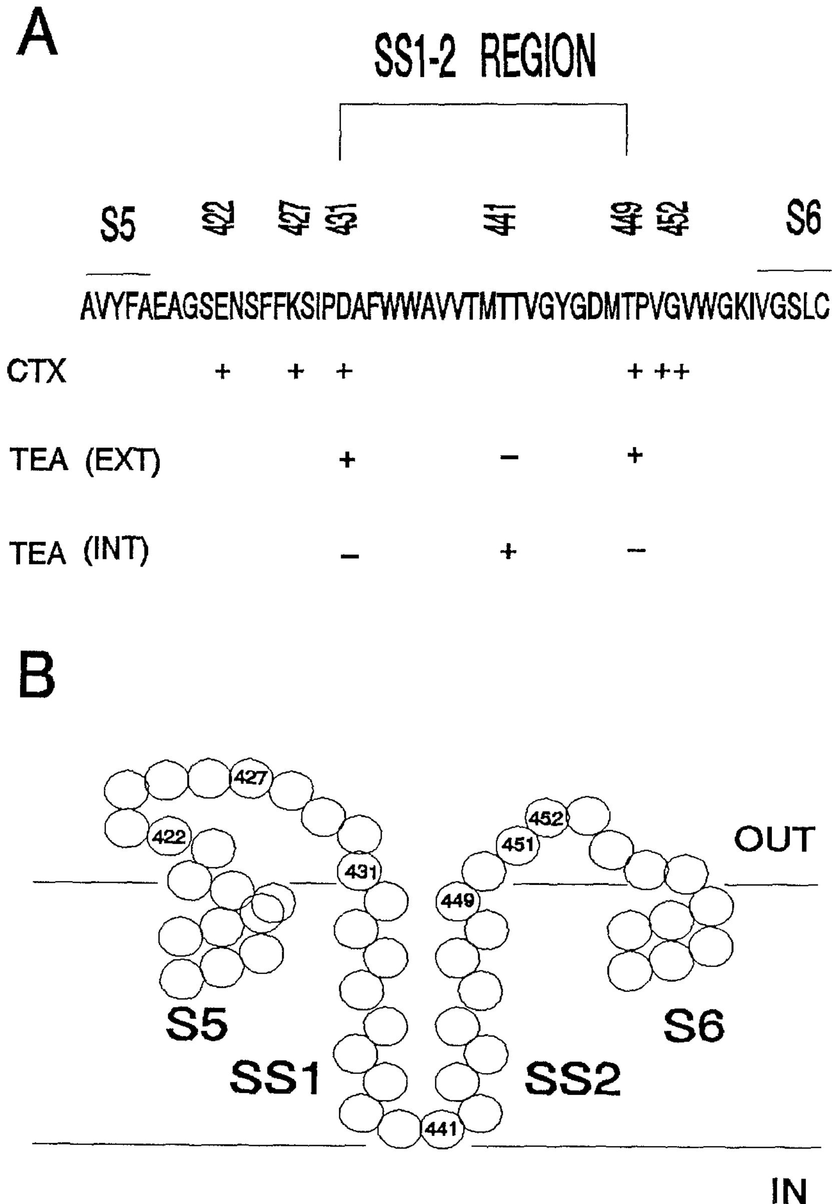

Fig. 2. (A) The sequence of the S5–S6 linker for a Shaker A-type K^+ channel subunit is shown in single-letter code. The stretch of amino acids in the middle of the linker is highly conserved among all known voltage-activated K^+ channels. The numbers above the sequence indicate the residue position in the Shaker H4 K^+ channel (Kamb *et al.*, 1987) where point mutations alter the sensitivity of the channel to CTX, external TEA, or internal TEA. A (+) indicates an effect and (−) indicates no effect. (B) The S5–S6 linker is proposed to dip into the membrane where it forms part of the ion-conductive pore. The two strands of the pore-forming hairpin are referred to as SS1 and SS2 according to the model of Guy (Guy and Conti, 1990).

Table I Effect of Mutations in the S5–S6 Linker of a Shaker K^+ Channel on Inhibition by CTX and TEA (Applied Internally and Externally)[a]

Position and mutation	K_i (CTX), nM	K_i (TEA Int), mM	K_i (TEA Ext), mM	K_i (TEA Ext), equivalent natural channels
Wildtype	3.7	0.7	30	
422				
E → D	4.1			
E → Q	14.5			
E → K	48		24	
427				
K → R	3.7			
K → N	0.55			
K → E	0.15		31	
431				
D → E	10			
D → N	> 1000			
D → K	> 1000	0.6	76	
441				
T → S		7.4	30	
449				
T → K	> 1000		> 200	RCK4 (> 100)
T → V			> 150	RCK5 (129)
T → Y	> 500	0.69	0.65	RCK1 (0.6)
T → Q	> 1000		> 150	
T → R			> 150	
451				
V → K	> 1000			
452				
G → R	9.5		30	
G → E	2.7			

[a] Inhibition constants are reported in nanomolar units for CTX and in millimolar units for TEA. Also shown are inhibition constants for external TEA inhibition of RCK K^+ channels. The Shaker K^+ channel data are from MacKinnon *et al.* (1990b), MacKinnon and Yellen (1990), and Yellen *et al.* (1990) and the RCK data are from Stühmer *et al.* (1989b).

amino acids connecting the fifth and sixth putative membrane-spanning segments—see Fig. 2A) influenced toxin block profoundly (MacKinnon and Miller, 1989b; MacKinnon *et al.*, 1990). Some mutations had effects that are consistent with a simple through-space electrostatic interaction (Table I), placing these amino acid residues in close proximity to the toxin binding site (positions 422, 427, and possibly 452). Mutations at another site suggested the possibility of a direct ionized hydrogen bond interaction between the toxin and the channel (position 431). Finally, some mutations had effects that were not explainable in terms of an obvious mechanism: perhaps they interfere sterically with the binding of the toxin molecule.

The charge-altering mutations that influence CTX inhibition form a very suggestive pattern. Just outside of S5 and S6, mutations affect CTX block. In the middle of the S5–S6 linker there is a stretch of 16 amino acids (between residues 431 and 449 shown in Fig. 2A) that are highly conserved among the voltage-activated K^+ channels; charge-altering mutations in this region result in the loss of detectable K^+ current. These results are consistent with a hypothetical model, proposed by Guy, for the membrane folding topology of a K^+ channel subunit (Guy and Conti, 1990). According to the model, the highly conserved stretch crosses the membrane twice by making a hairpin turn. The two segments of the hairpin are referred to as SS1 and SS2 for short segments 1 and 2 (Fig. 2B). When four subunits are put together in the model, the residues of four short segment pairs, one contributed by each of the subunits, line the pore. The model predicts that drastic mutations in the SS1–2 region (the conserved stretch between 431 and 449) that forms the narrow pore may interfere with functional expression of the channel. The model also places the residues that flank the SS1–2 region in the channel's outer mouth, where they would be expected to interact with a bound toxin molecule.

External TEA: A Higher Resolution Probe

The SS1–2 pore model gained further support when some of the mutations that influence CTX inhibition were also found to affect inhiition by extracellular tetraethylammonium ion (TEA) as well as the conduction of K^+ ions through the pore (MacKinnon and Yellen, 1990). The K^+ channel inhibitor TEA is small compared to CTX: it has roughly the same dimensions as a fully hydrated K ion and is thought to enter into and block the ion-conductive pore (Armstrong, 1975). From the extracellular side, TEA probably enters only a very short distance into the pore since its inhibition is affected weakly by membrane voltage. Mutations involving the amino acid residue at each end of the SS1–2 region in the Shaker A-type K^+ channel (at positions 431 and 449) were found to influence extracellular TEA inhibition, while mutations further removed (positions 422, 427, 452) did not (Table I). These findings are in complete harmony with the hypothesis that the SS1–2 region forms the narrow pore by looping into the membrane, since such an arrangement places residues 431 and 449 near the external entryway to the pore, where TEA is thought to bind.

Mutations involving residue 449 affect TEA inhibition dramatically. Moreover, random variation at this position among some naturally occurring voltage-activated K^+ channels appears to account for most of their differences in sensitivity to external TEA. When the threonine residue at position

449 in the wild-type Shaker A-type K^+ channel was replaced by the corresponding amino acid of a different K^+ channel, the Shaker channel acquired the approximate external TEA sensitivity of that other channel. (See the RCK K^+ channels in Table I.) Of particular note is the high external TEA sensitivity of those channels with a tyrosine residue at the 449 equivalent position (Stühmer *et al.*, 1989b; Frech *et al.*, 1989; Swanson *et al.*, 1990; MacKinnon and Yellen, 1990). The striking correspondence between the amino acid residue at 449 and a channel's sensitivity to external TEA, even among K^+ channels with many differences elsewhere, argues that a blocking TEA molecule probably interacts intimately with that residue. The probable location of residue 449 in the narrow external entryway to the pore is consistent with the finding that some mutations at this position affected K^+ ion conduction. In particular, lysine, arginine, or glutamine substitution at 449 in the Shaker A-type K^+ channel reduced the single-channel conductance. A naturally occurring K^+ channel (RCK4) with a lysine at this position also has a smaller single-channel conductance (Stühmer *et al.*, 1989b).

Internal TEA: Finding the Intracellular Entryway

If the SS1–2 region extends across the membrane as a hairpin loop as depicted in Fig. 2B, then some of the residues must be located close to the channel's intracellular mouth. This prediction was tested by asking if conservative mutations between 431 and 449 in the Shaker A-type K^+ channel affect the channel's interaction with a pore-blocking agent that acts from the inside (Yellen *et al.*, 1990). TEA was again used since it also blocks from the inside. Its site of action is near the inner mouth of the channel and is distinct from the external TEA inhibition site. A threonine-to-serine mutation at position 441, located in the middle of the SS1–2 region, specifically affected inhibition by internal TEA. The mutation weakened block by a factor of 10 but left other properties of the channel, including voltage-dependent gating, single-channel conductance, and inhibition by external TEA, completely intact. Furthermore, the mutations that had been shown to influence external TEA inhibition (at positions 431 and 449) did not affect inhibition by internal TEA. These findings point to position 441 as being near the intracellular entryway to the ion-conductive pore.

Residue 441 interacts with internal TEA and yet it is separated by only eight amino acid residues from a site that interacts with external TEA (position 449). These findings are surprising when one considers the voltage dependence of external and internal TEA blockade. From the extracellular side, TEA traverses only about 5% of the transmembrane electric potential when it reaches its inhibition site, and from the intracellular side it traverses only 15%. Most of the transmembrane electric potential must therefore change over a stretch of only eight amino acids (between residues 441 and

449). As an α-helix, eight amino acids span a distance of only 12 Å, or less than half the thickness of a cell membrane. Perhaps the channel has a short, narrow pore that connects wide inner and outer vestibules which are made of protein contributed by other parts of the channel molecule. In this case the channel would have an hourglass shape, reminiscent of the channel structure proposed by Latorre and Miller for large-conductance (Maxi) K^+ channels (Latorre and Miller, 1983). An alternative possibility, suggested by Guy (Guy and Conti, 1990), is that the SS1–2 region forms a β-hairpin turn. A β-strand of eight amino acids is about 27 Å long and therefore has nearly the same length as a 20 amino acid α-helix. (In Guy's model, two antiparallel β-strands from each subunit form an eight-stranded β-barrel with a central cavity.)

At present we can only speculate, but the longer pore afforded by a β-conformation seems to be more compatible with what we already know about the ion conductive pore of many K^+ channels. Voltage-activated K^+ channels are multi-ion channels that allow at least three K^+ ions to line up in single file (Hodgkin and Keynes, 1955; Spalding *et al.*, 1981; Begenisich and DeWeer, 1980). For electrostatic reasons alone, a short conduction pore seems implausible. Further, streaming-potential measurements carried out in a Ca^{2+}-activated K^+ channel demonstrated the obligatory movement of two water molecules with each K ion (Alcayaga *et al.*, 1989). Three K ions and six water molecules in a queue span at least 20 Å. Finally, a study of bis-quaternary ammonium ion block of a Ca^{2+}-activated K^+ channel showed that 27% of the transmembrane electric potential falls across a physical distance of about 10 Å (Villarroel *et al.*, 1989). Assuming a constant field across the narrow region of the pore, 80% of the transmembrane electric potential will fall across a distance of about 30 Å. Each of these arguments alone can be criticized, but together they provide substantial evidence that the highly K^+ selective channels, like the voltage and Ca^{2+}-activated K^+ channels, have a pore that is around 30 Å long[3].

The combined molecular-biological/electrophysiological approach has led to the identification of one region, between S5 and S6, that appears to be intimately involved in the formation of the ion-conductive pore. We have not yet begun to decipher the molecular mechanisms of ion permeation and selectivity, but future focus on this region of the molecule should be fruitful. Ultimately, we will need to know the pore structure before we can fully understand how the protein creates an environment where several K ions can line up in a confined space and Na ions cannot. But even before we know the

[3]The streaming-potential measurements and bis-quaternary ammonium blocker studies were carried out on Ca^{2+}-activated K^+ channels. However, in many respects the ion conduction properties of voltage-activated and Ca^{2+}-activated K^+ channels are very similar (Yellen, 1987).

pore's structure, the mutagenesis-function approach should enable us to identify residues that control ion selectivity, and to entertain models of how functional groups may interact with ions as they diffuse through the pore.

The Inactivation Gate

The Ball and Chain Proposal for Inactivation

Many voltage-activated cation channels open in response to membrane depolarization and then they spontaneously shut. The spontaneous closing process is called inactivation and for some ion channels, including Na^+ channels and A-type K^+ channels, the process is very rapid and complete in only tens of milliseconds. These ion channels are said to undergo fast inactivation. Fast inactivation in Na^+ channels was studied in detail by Armstrong and Bezanilla (1977); several features of the process led them to propose that a cytoplasmic domain of the channel acts as a gate by swinging into and occluding the pore after the channel opens. The idea is known as the "ball and chain" model of inactivation.

A Ball and Chain in a Shaker A-Type K^+ Channel

Shaker A-type K^+ channels undergo fast inactivation, and in many respects the process is very similar to that observed in Na^+ channels. A channel must open before it can inactivate (Armstrong and Bezanilla, 1977; Hoshi *et al.*, 1990). There is little or no intrinsic voltage dependence to inactivation, as one might expect if a non-membrane-spanning component acts as a physical gate. Fast inactivation is abolished in A-type K^+ channels, as in Na^+ channels, by exposing the cytoplasmic face to proteolytic enzymes (Armstrong *et al.*, 1973; Hoshi *et al.*, 1990). This finding suggests that an inactivation gate can be removed, leaving the channel otherwise intact. Finally, pore-blocking inhibitors added to the inside of K^+ channels which do not rapidly inactivate (Armstrong, 1971), or to trypsin-treated A-type K^+ channels, mimic the inactivation process. These properties are consistent with a ball and chain mechanism. In further support, it was shown recently that internal TEA inhibits the inactivation process in a competetive manner, just as one might expect if the inactivation gate (or ball) binds at a site in the channel's inner mouth (Choi *et al.*, 1991).

Alternatively spliced Shaker A-type K^+ channels with different amino terminal cytoplasmic domains display different rates of inactivation (Timpe *et al.*, 1988). This observation focused attention on the amino terminus as a potential structural component of the fast inactivation process. Alterations of the amino terminus were found to affect inactivation. A systematic and

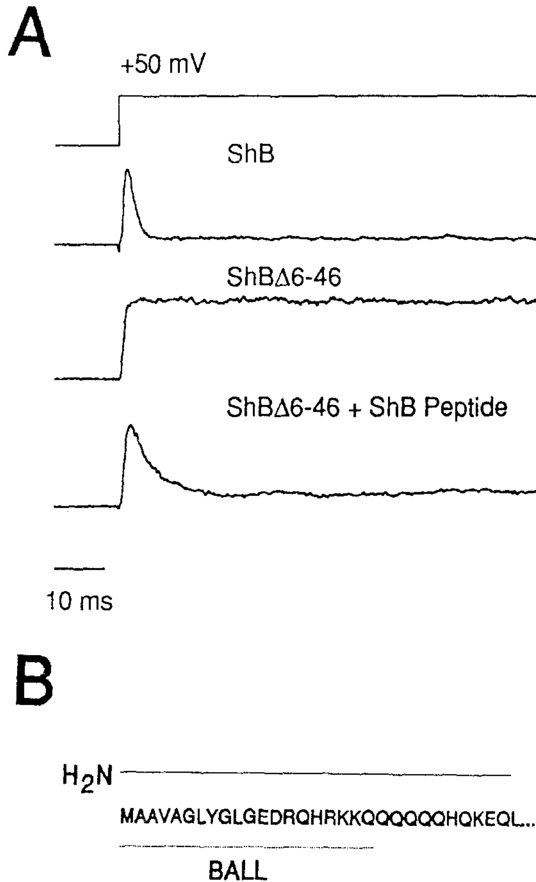

Fig. 3. The amino terminus of a Shaker A-type K^+ channel polypeptide acts as a cytoplasmic blocker of the channel. (A) The three current traces were recorded during depolarization (to + 50 mV) of an inside-out oocyte membrane patch containing many Shaker K^+ channels. The Shaker B K^+ channel (ShB; Schwarz *et al.*, 1988) displays the characteristic inactivation of an A-type K^+ channel (the channels inactivate rapidly after opening). A mutation that deletes amino acid residues 6 to 46 (ShB 6–46) removes inactivation. Addition of 100 μM synthetic peptide corresponding to the first 20 amino acids of the Shaker B K^+ channel restores inactivation in the noninactivating deletion mutant. (B) The sequence of the amino terminus of the Shaker B K^+ channel. The proposed ball-forming region is underlined. (Data provided by Richard Aldrich.)

thorough set of mutations enabled Aldrich and colleagues to identify two functionally distinct segments (Hoshi *et al.*, 1990). One segment is defined by the first 20 amino acids and the other by the following 60 or so (Fig. 3B). Deletions and even point mutations involving the first 20 amino acid residues either eliminated or substantially slowed the rate of inactivation. The first half of the 20 amino acid stretch is hydrophobic and the second half is

cationic: both halves are important since alteration of either adversely affects inactivation. In contrast, mutations involving residues 20 to about 80 had the tendency either to slow or accelerate the transition to the inactivated state, depending upon whether a mutation lengthened (slowed inactivation) or shortened (accelerated inactivation) this stretch of amino acids.

On the basis of these findings, Aldrich and colleagues proposed that the amino terminus of a Shaker A-type K^+ channel may correspond to the structural inactivation gate, or ball and chain. The critical effects of mutations in the first 20 amino acid stretch suggested that this segment may form the ball, or structure, which might interact in a specific manner with the inner mouth of the pore. They proposed that the hydrophobic region may form the core of a cationic inactivation ball. The idea was critically tested by asking if a synthetic peptide, corresponding to the first 20 residues, would be competent to induce inactivation when exposed to the inside of mutant, noninactivating Shaker A-type K^+ channels (Zagotta *et al.*, 1990). (See Fig. 3.) Not only did the free peptide functionally reconstitute the inactivation process, but it also displayed the correct molecular specificity: variant peptides with sequences mimicking the amino terminus of noninactivating mutant channels did not induce inactivation. These experiments provide very strong evidence that the first 20 amino acids of a Shaker A-type K^+ channel act as a tethered, cytoplasmic open-channel blocker.

The transitions that occur between gating (conformational) states of an ion channel protein may be difficult to understand in general. But for the case of fast inactivation in Shaker A-type K^+ channels, an important structural element has been identified, and a clear picture of the underlying molecular mechanism has been provided.

Summary and Concluding Remarks

Molecular-genetic techniques have revolutionized mechanistic ion channel research. We are now able to ask questions that we could only dream about just a few years ago and, consequently, new information is coming in at an extraordinary rate. For voltage-activated K^+ channels in particular, the impact of our new abiilty to manipulate ion channel molecules has had profound effects. Over the past two years, three important structural conclusions have been reached and these are depicted in Fig. 4. First, voltage-activated K^+ channels are tetramers. Second, a pore-forming region has been identified, and surprisingly, it is not one of the six stretches that presumably form the hydrophobic core of the K^+ channel subunit. It appears that a relatively short stretch of residues in the "linker" between S5 and S6 dips into

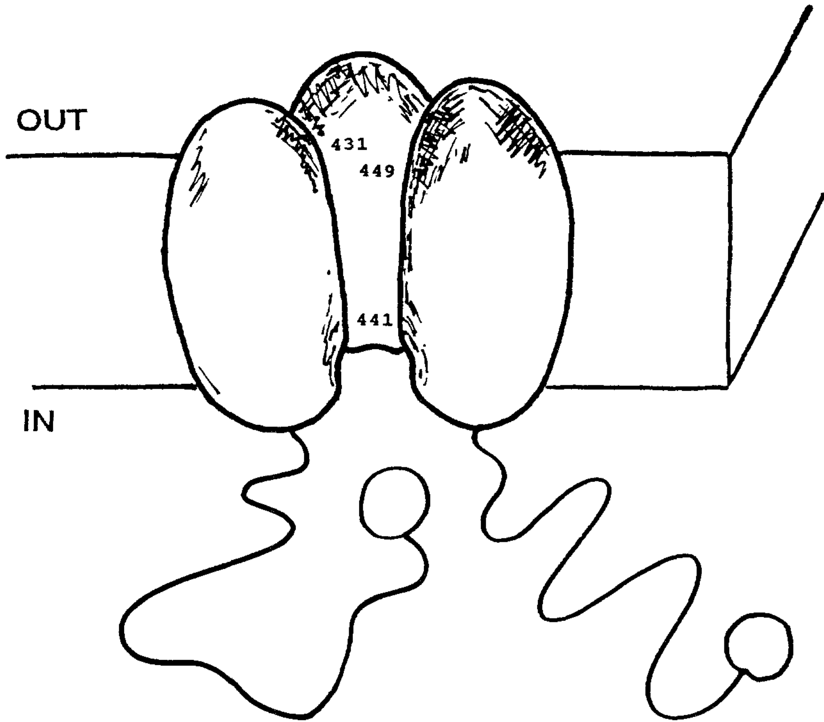

Fig. 4. A cartoon of a voltage-activated K^+ channel with one subunit removed. The channel is a tetramer. Residues 431 and 449 (of the Shaker B or Shaker H4 A-type K^+ channels) are near the external entryway to the pore and 441 is near the internal entryway. The amino terminus of each subunit can block the pore and thus inactivate the channel from the inside.

the membrane (but may be completely surrounded by protein) to form part of the pore lining. Third, the molecular mechanism as well as an important underlying structural component (an amino terminal inactivation gate) involved in fast inactivation gating in a Shaker A-type K^+ channel has been elucidated.

The next few years will certainly be an exciting time in the development of our understanding of K^+ channel mechanisms. The examples described here are only the beginning; the picture in Fig. 4 will be modified many times in the near future. Perhaps we will soon know which amino acid side groups point toward the ion-conductive pore and which ones point away. It will be interesting to see if Ca^{2+}-activated and inward rectifier K^+ channels have a conserved SS1-2 like sequence, as we might expect on the basis of their similar ion-conduction properties. Hopefully we will begin to identify domains that are important for other gating steps, like those leading to channel opening, and those responsible for slow inactivation. Maybe we will even begin to

understand how that highly unusual sequence of intermittent hydrophobic and cationic amino acid residues, the S4, is coupled to voltage-dependent gating. Certainly, many questions will remain unanswered until we can actually look at the structures of ion channels. But hopefully we will soon have direct structural information—and then the fun will really begin.

Acknowledgments

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References

Alcayaga, C., Cecchi, X., Alvarez, O., and Latorre, R. (1989). *Biophys. J.* **55**, 367–372.
Anderson, C., MacKinnon, R., Smith, C., and Miller, C. (1988). *J. Gen. Physiol.* **91**, 317–333.
Armstrong, C. M. (1971). *J. Gen. Physiol.* **58**, 413–437.
Armstrong, C. M. (1975). *Potassium Pores of Nerve and Muscle Membranes* (Eisenman, G., ed.), Marcel Dekker, New York, pp. 325–358.
Armstrong, C. M., and Bezanilla, F. (1977). *J. Gen. Physiol.* **70**, 567–590.
Armstrong, C. M., and Hille, B. (1972). *J. Gen. Physiol.* **59**, 388–400.
Armstrong, C. M., Bezanilla, F., and Rojas, E. (1973). *J. Gen. Physiol.* **62**, 375–391.
Begenisich, T. B., and DeWeer, P. (1980). *J. Gen. Physiol.* **76**, 83–98.
Catterall, W. A. (1988). *Science* **242**, 50–61.
Choi, K. L., Aldrich, R. W., and Yellen, G. (1991). *Proc. Natl. Acad. Sci. USA*. (In press).
Christie, M. J., North, R. A., Osborne, P. B., Douglass, J., and Adelman, J. P. (1990). *Neuron* **4**, 405–411.
Frech, G. C., VanDongen, A. M. J., Schuster, G., Brown, A. M., and Joho, R. H. (1989). *Nature (London)* **340**, 642–645.
Guy, H. R., and Conti, F. (1990). *Trends Neurosci.* **13**, 201–206.
Hille, B. (1984). *Ionic Channels of Excitable Membranes*, Sinauer Associates, Sunderland, Massachusetts, pp. 426.
Hodgkin, A. L., and Keynes, R. D. (1955). *J. Physiol.* **128**, 61–88.
Hoshi, T., Zagotta, W. N., and Aldrich, R. W. (1990). *Science* **250**, 533–538.
Isacoff, E. Y., Jan, Y. N., and Jan, L. Y. (1990). *Nature (London)* **345**, 530–534.
Kamb, A., Iverson, L. E., and Tanouye, M. A. (1987). *Cell* **50**, 405–413.
Latorre, R., and Miller, C. (1983). *J. Membr. Biol.* **71**, 11–30.
MacKinnon, R. (1991). *Nature (London)*, **350**, 232–235.
MacKinnon, R., and Miller, C. (1988). *J. Gen. Physiol.* **91**, 335–349.
MacKinnon, R., and Miller, C. (1989a). *Biochemistry* **28**, 8087–8092.
MacKinnon, R., and Miller, C. (1989b). *Science* **245**, 1382–1385.
MacKinnon, R., and Yellen, G. (1990). *Science* **250**, 276–279.
MacKinnon, R., Reinhart, P. H., and White, M. M. (1988). *Neuron* **1**, 997–1001.
MacKinnon, R., Latorre, R., and Miller, C. (1989). *Biochemistry* **28**, 8092–8099.
MacKinnon, R., Hegingotham, L., and Abramson, T. (1990). *Neuron* **5**, 767–771.
Miller, C. (1982). *J. Gen. Physiol.* **79**, 869–891.
Miller, C. (1988). *Neuron* **1**, 1003–1006.
Miller, C., Moczydlowski, E., Latorre, R., and Phillips, M. (1985). *Nature (London)* **313**, 316–318.
Pongs, O., Kecskemethy, N., Muller, R., Kreh-Jentgens, I., Baumann, A., Kiltz, H. H., Canal, I., Llamazares, S., and Ferrus, A. (1988). *EMBO J.* **7**, 1087–1096.

Ruppersberg, J. O., Schröter, K. H., Sakmann, B., Stocker, M., Sewing, S., and Pongs, O. (1990). *Nature (London)* **345**, 535–537.
Schwarz, T. L., Tempel, B. L., Papazian, D. M., Jan, Y.-N., and Jan, L.-Y. (1988). *Nature (London)* **331**, 137–142.
Spalding, B. C., Senyk, O., Swift, J. G., and Horowicz, P. (1981). *Am. J. Physiol.* **241**, c68–c75.
Stühmer, W., Conti, F., Suzuki, H., Wang, X., Nada, M., Yahagi, N., Kubo, H., and Numa, S. (1989a). *Nature (London)* **339**, 597–603.
Stühmer, W., Ruppersberg, J. P., Schroter, K. H., *et al.* (1989b). *EMBO J.* **8**, 3235–3244.
Swanson, R., Marshall, J., Smith, J. S., Williams, J. B., Boyle, M. B., Folander, K., Luneau, C. J., Antanavage, J., Oliva, C., Buhrow, S. A., Bennett, C., Stein, R. B., and Kaczmarek, L. K. (1990). *Neuron* **4**, 929–939.
Tempel, B. L., Papazian, D. M., Schwarz, T. L., Jan, Y. N., and Jan, L. Y. (1987). *Science* **237**, 770–775.
Timpe, L. C., Jan, Y. N., and Jan, L. Y. (1988). *Neuron* **1**, 659–667.
Villarroel, A., Alvarez, O., Oberhauser, A., and Latorre, R. (1989). *Pflugers Arch.* **413**, 118–126.
Yellen, G. (1987). *Ann. Rev. Biophys. Biophys. Chem. 1987*. 227–246.
Yellen, G., Jurman, M., Abramson, T., and MacKinnon, R. (1991). *Science* **251**, 939–942.
Zagotta, W. N., Hoshi, T., and Aldrich, R. W. (1990). *Science* **250**, 568–571.