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# The Structure and Function of the Shaker Voltage-Gated Potassium Channel: A Mutational Analysis Examining Voltage-Dependent Gating, Potassium Ion Permeation, and the TEA and Barium Ion Binding Sites

by

George Arthur Lopez

**DISSERTATION**

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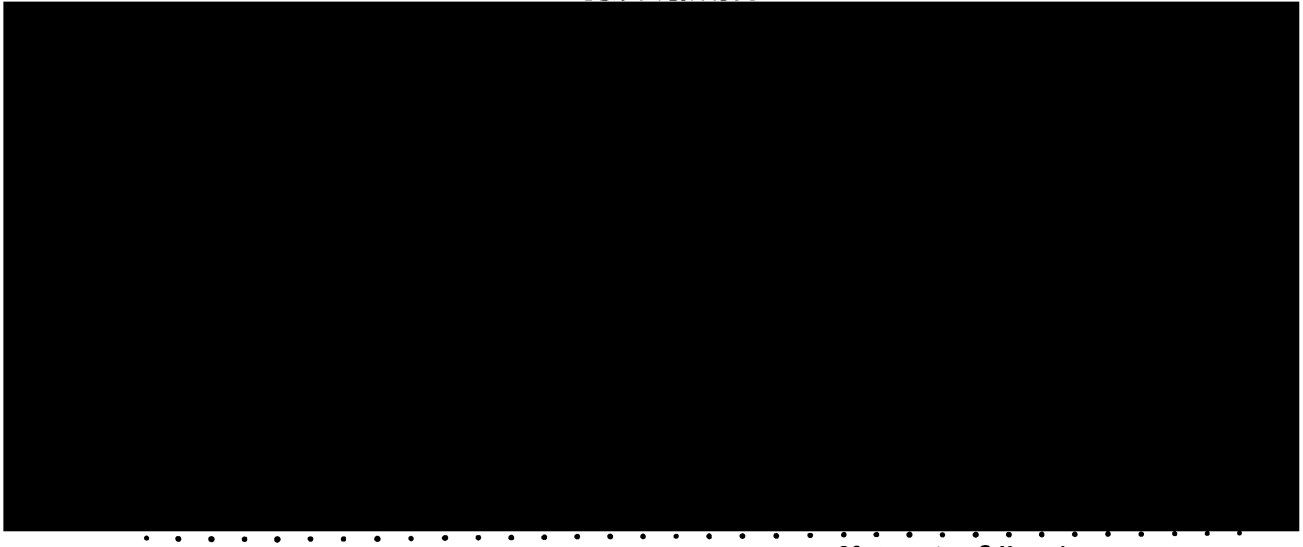

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## DEDICATION

This thesis is dedicated to

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## PREFACE

I am grateful to all of the members of the Jan lab who made my stay both intellectually stimulating, challenging, and most of all fun. I would like to thank: Tim Baldwin, Tony Collins, Tom Crowley, Ed Giniger, Udi Isacoff, Bill Kimmerly, Yoshihiro Kubo, Min Li, Diane Papazian, Eitan Reuveny, Thomas Schwarz, Morgan Sheng, Paul Slesinger, Les Timpe, Mei-Ling Tsaur for their scientific expertise and friendship; Ming Guo and Michelle Solis, my rotation students who did some of the scut work for me (eg. sequencing and mini-preps); Denise Mulhrad and Sharon Freid for their excellent technical assistance; Kathy Prewitt and Barbara Bannerman for last minute orders; Mark Kirschner for use of the frogs; Rick Aldrich for the 6-46 deletion mutation of *Shaker* B; Rolf Joho and Buzz Brown for the NGK2 clone; Ira Clark, Dan Doherty, and Michelle Rhyu for just being the fellow graduate students in the lab; and Eric Kandel for communication of sequence data prior to publication.

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George Arthur Lopez

**The Structure and Function of The *Shaker* Voltage-Gated Potassium Channel**

**A Mutational Analysis Examining Voltage-Dependent Gating,**

**Potassium Ion Permeation, and the TEA**

**and Barium Ion Binding Sites**

$K^+$ channels represent a diverse group of ion channels that have been found in almost all eukaryotic cells. The functions of these channels depend on the specific ways a $K^+$ channel opens and closes, and on its selective permeation by $K^+$ ions. A wide range of these channel properties has been observed and is crucial for subserving different physiological functions.

How does the channel respond to changes in membrane potential and open? How does a $K^+$ channel discriminate between $K^+$ ions and other cations? With experiments involving site-directed mutagenesis and patch-clamping single-channels, I have identified and localized structural domains and single amino-acids that are critical for these specific channel functions.

The S4 domain contains positively charged residues at every third position interspersed with hydrophobic residues and has been theorized to serve as the channels voltage sensor. To explore the role of the S4 domain, I created hydrophobic substitution mutations and discovered that they had local effects, limited to the hydrophobic interactions between the S4 domain and its immediate surrounding. These mutations caused very large shifts in

the voltage-dependence of channel activation suggesting that even the mildest alteration of hydrophobic interactions in the S4 domain alter the relative stabilities of the open and the closed states. In contrast, similar mutations of residues in the other hydrophobic domains had little effects on the voltage-dependence of channel activation. The S4 sequence therefore, is unique and critical in its involvement in voltage-dependent gating.

Potassium channels also exhibit remarkable selectivity among the physiologically relevant ions: $K^+$ goes through the pore ten thousand times more readily than sodium which has a smaller crystal radius. This tremendous selectivity is achieved with little compromise in the rate of $K^+$ permeation: more than a million $K^+$ ions go through the pore in one second. In order to uncover the pore lining structure, I created mutations in the sixth transmembrane domain and discovered alterations in the single-channel conductance, the binding of the open channel blockers tetraethylammonium and barium ions, as well as modifications in the channels ability to discriminate between ions of different size. These effects are expected if the sixth transmembrane domain contributes amino acids to the pore structure. These mutations identify and localize part of the inner vestibule and pore lining structure of the *Shaker* $K^+$ channel to the sixth transmembrane domain

Zach W. Hall

Zach Hall, Ph.D.

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# CHAPTER I

# INTRODUCTION

No discussion about ionic channels and excitable membranes would be complete without referring to the classic papers of Hodgkin and Huxley (Hodgkin et al., 1952; Hodgkin and Huxley, 1952a; Hodgkin and Huxley, 1952b; Hodgkin and Huxley, 1952c; Hodgkin and Huxley, 1952d). They described membrane currents recorded from voltage-clamped squid axons and analyzed the underlying ionic conductances in terms of a simple yet elegant mathematical model. They proposed that the total ionic current that flowed through the axon membrane could be dissected into two components, one carried by sodium and the other by potassium ions (Hodgkin and Huxley, 1952a), and that the magnitudes of these conductances were determined by the membrane potential (Hodgkin and Huxley, 1952b). Their quantitative description of gating suggested the presence of a voltage-dependent gating mechanism. Beginning with the time course of potassium current that was evoked by step depolarizations, Hodgkin and Huxley (Hodgkin and Huxley, 1952d) proposed an empirical model that described the time course of the potassium conductance in terms

of a dimensionless variable *n*, and for the sodium conductance by the variables *m* and *h*. It was with these variables that Hodgkin and Huxley derived the mathematical equations describing the ionic conductances of the squid giant axon and from this description emerged the idea of the voltage-dependent ion channel.

In the days of Hodgkin and Huxley, it was still unknown in terms of the pathway, how ions crossed the membrane. Hodgkin and Huxley considered both carriers and pores as the possible transport mechanisms. Carriers were thought to bind the ions on one side of the membrane, ferry them through the membrane, and release them on the other side. Central to the Hodgkin-Huxley concept was the separation of the total ionic currents into sodium and potassium conductances (Hodgkin and Huxley, 1952d), each independent of the other. They were not, however, able to specify whether the two permeation pathways were physically distinct entities or what the physical nature of these pathways were. That there were two distinct pathways came from later kinetic and pharmacological experiments.

The effects of toxins such as tetrodotoxin (Narahashi et al., 1964, Moore et al., 1967) and saxitoxin (Narahashi et al., 1967) supported the

view that the pathways were physically distinct. These toxins were found to affect only the sodium conductance with no effect on the potassium conductance. This idea was further supported by the use of potassium selective drugs such as tetraethylammonium (TEA) (Lorente de No, 1949; Shanes, 1958; Hille, 1967). Additional experiments providing evidence against the single-channel hypothesis used pronase to remove sodium channel inactivation without affecting the potassium conductance (Rojas and Armstrong, 1971; Armstrong et al., 1973; Rojas and Rudy, 1976). Later, several theories arose about the nature of the ionic pathways. One theory was that the barrier to ion permeation was formed by the protein and that the ion pathway was through the lipid. It wasn't until experiments with artificial bilayers (Mueller et al., 1962) and with molecules such as valinomycin and the gramicidin A pore (Finkelstein and Anderson, 1981; Hladky and Haydon, 1970) that the idea of an ion pore was more widely accepted. Direct evidence for separate ionic pathways began with the biochemical solubilization of a tetrodotoxin-binding protein (Henderson and Wang, 1972) and the demonstration that this protein had the expected properties for the sodium channel (reviewed in Miller, 1986). Later, the patch-clamp technique (Neher and Sakmann, 1976; Hamill et al., 1981) was able to demonstrate single functioning ionic channels which never revealed

any spontaneous changes in the identity of the permeating ion when more than one permeant ion was present. Probably the most incontrovertible evidence for the idea that sodium and potassium channels are indeed distinct entities came from the cloning of genes encoding the channels (Noda et al., 1984; Papazian et al., 1987; Kamb et al., 1988; Pongs et al., 1988).

## POTASSIUM CHANNELS

Potassium channels form the largest and most diverse class of ion channels. They are found in both electrically excitable and non-excitable cells in all tissues of the body. They have been identified in species as primitive as yeast and bacteria (Anderson et al., 1992). There are well over a dozen distinguishable types of potassium channels which differ in their gating and regulation. Common characteristics they share are their high unitary transport rate and ion selectivity. These ion channels can exhibit conduction rates ranging from 10 to 100 million potassium ions per second while retaining the capacity to exclude sodium, magnesium, calcium, and chloride ions from passing. A common characteristic of voltage-activated ion channels is the presence of an S4 sequence which has been found in all

voltage-activated channels cloned thus far (Jan and Jan, 1990b). The S4 sequence is a presumed $\alpha$-helix with positively charged residues spaced every three residues in series and is proposed to act as the voltage sensor of these channels (Hille, 1984; Catterall, 1986; Catterall, 1988; Guy, 1989; Stuhmer et al., 1989; Papazian et al., 1991). Given these common features, it is likely that potassium channels have evolved "functional protein motifs" governing their similarities. To aid in the understanding of how potassium channels function, we would like to learn more about their common features.

## MOLECULAR BIOLOGY OF VOLTAGE-GATED POTASSIUM CHANNELS

The molecular biology of potassium channels has been extensively reviewed recently (Jan and Jan, 1990a; Jan and Jan, 1992b; Pongs, 1992). Voltage-gated potassium channels are the most diverse and ubiquitous of the voltage-gated ion channels (Rudy, 1988), which include the family of sodium, calcium, and chloride channels. Potassium channels can be grouped into four different classes: (a) the delayed rectifier current, (b) the transient or A-type current, and (c) the inward rectifier current. The fourth class of

potassium channels is a diverse group but members all have in common the requirement of a ligand as well as a change in membrane potential for activation and the class is referred to as ligand-gated potassium channels. For this class, a change in membrane potential alone is insufficient for channel gating. Also required are specific channel ligands such as calcium (Meech and Standen, 1975; Latorre et al., 1984), sodium (Kameyama et al., 1984), ATP (Noma, 1983; Ashcroft, 1988; Ashcroft and Ashcroft, 1990), or G Proteins (Logothetis et al., 1987; Yatani et al., 1987; Brown, 1990). Members from all four classes of potassium channels have been cloned, sequenced, and expressed (Frech et al., 1989; Timpe et al., 1988b; Ho et al., 1993; Kubo et al., 1993a; Adelman et al., 1992; Butler et al., 1993; Kubo et al., 1993b; Dascal et al., 1993).

Because high affinity ligands for voltage-gated potassium channels were not available as they were for the acetylcholine receptor (Raftery et al., 1980), a biochemical approach to potassium channel protein purification and subsequent peptide sequencing was not possible. There was however a behavioral mutant of the fruit fly *Drosophila melanogaster* called *Shaker*. This mutant fly was called shaker because under ether anesthesia, the mutant flies would shake their appendages violently (Catsch, 1944; Kaplan

and Trout, 1969). The *Shaker* mutant exhibited electrophysiological abnormalities in action potential firing as well as in neurotransmitter release at the larval neuromuscular junction. The first indication of a potassium channel defect emerged in a study of neuromuscular transmission in *Shaker* mutant larvae muscle fibers (Jan et al., 1977). Specifically, the *Shaker* nerve fibers showed broadened action potentials and multiple firings (Tanouye et al., 1981) and the neuromuscular junctions exhibited prolonged transmitter release from the nerve terminals which could be mimicked by treating the wild-type larval preparation with a potassium channel blocker, 4-AP (Jan et al., 1977). All of this suggested that a gene within the *Shaker* locus encoded for a structural component of a potassium channel.

The *Shaker* mutations affect a fast, transient potassium current. It was found that different *Shaker* alleles reduce, eliminate, or alter the transient potassium current (Salkoff and Wyman, 1981) without affecting other currents (Wu and Haugland, 1985). This was discovered by voltage-clamp analysis of membrane currents in larval muscle fibers as well as with single-channel analyses. It was proposed that the *Shaker* locus encoded a structural component of the transient A-type potassium channel (Tanouye et al., 1981; Salkoff 1983).

With the use of molecular biological techniques, the *Shaker* locus was cloned (Papazian et al., 1988). A DNA fragment that hybridized nearby to the *Shaker* locus was used to begin a chromosomal walk through the locus (Papazian et al., 1987). Overlapping clones were isolated from genomic DNA libraries until over 200,000 bases of genomic DNA had been isolated. To localize the *Shaker* gene, several *Shaker* mutations caused by chromosomal rearrangements were mapped and found to be within a 65,000 base region of the cloned DNA. DNA from this region was then used to screen cDNA libraries (Papazian et al., 1987).

The sequences of the *Shaker* cDNAs were subsequently determined (Tempel et al., 1987). The *Shaker* gene encodes an integral membrane protein of more than 600 amino acids. The hydropathy analysis shows that the protein has a central hydrophobic core with hydrophilic domains at the amino and carboxyl termini. The central hydrophobic core contains six hydrophobic domains large enough to span the membrane in an alpha helical configuration. The *Shaker* protein shares significant homology with voltage-gated sodium and calcium channels, especially in the region of the S4 domain (Noda, 1984; Tanabe et al., 1987).

After the cDNAs were subsequently cloned, it was discovered that these cDNAs did not encode only one type of potassium channel protein but several closely related ones (Iverson et al., 1988; Timpe et al., 1988a; Timpe et al., 1988b; Stocker et al., 1990). Considerable molecular analysis has indicated that the *Shaker* cDNAs are derived from a large transcription unit, approximately 150,000 nucleotide bases in length, that contain at least 23 exons. Alternative splicing pathways and alternative transcription start sites lead to the synthesis of several different mRNAs encoding at least 10 different potassium channel subunits (Reviewed by Jan and Jan, 1990a; Pongs, 1992). The variant exon composition of alternative *Shaker* mRNAs results in the synthesis of proteins that share a common core region. The core region is highly conserved among potassium channels. The core region consists of five hydrophobic segments S1, S2, S3, S5 and S6, a positively charged domain S4, and a domain originally designated H5 which has hydrophilic features (Figure 1). The domains S1 through S6 are thought to traverse the membrane (Schwarz et al., 1988; Tempel et al., 1988; Guy and Conti, 1990), whereas the H5 domain (also named SS1-SS2, P-region; Guy and Conti, 1990; Hartmann et al., 1991) is thought to traverse the membrane as an anti-parallel beta sheet structure (see below) (Yool and Schwarz, 1991; Yellen et al., 1991; Hartmann et al., 1991) (Figure 2). The

core region is flanked by variant amino and carboxyl terminal sequences which give the different *Shaker* variants their distinctive electrophysiological properties. Five different amino termini and two different carboxy termini have been characterized from the *Shaker* locus in *Drosophila*.

Using low stringency hybridization techniques and polymerase chain reaction, numerous potassium channel genes have been isolated from a variety of species from the squid up to human. Most of the potassium channels cloned are from the voltage-gated potassium channel family. The rat genome for example, contains a gene family that encodes several highly homologous potassium channels (RCK1 to RCK9) that are closely related to the *Shaker* channel (Stuhmer et al., 1989b; Betsholtz et al., 1990). Each protein assembles into a homomultimer that expresses a distinct voltage-gated potassium channel with characteristic electrophysiological and pharmacological properties. Using *Shaker* derived oligonucleotide probes and homology screening, three other closely related gene families have been identified in *Drosophila*. These were named Shab, Shaw, and Shal (Shaker (Kv1.x), Shab (Kv2.x), Shaw (Kv3.x), Shal (Kv4.x); See Chandy, 1991 for a simplified potassium channel nomenclature) (Butler et al., 1989; Wei et al., 1990). The proteins encoded by these genes all have three domains, the

amino and carboxy termini, and the hydrophobic core. The core is approximately 70% conserved for members of each subfamily and approximately 40% conserved among different subfamilies.

Each potassium channel protein is encoded by separate genes. As with *Drosophila*, there is also alternative splicing of mammalian potassium channel genes creating an even more diverse group of potassium channels. Unlike the mammalian RCK family of potassium channels, the Raw family (named after the *Drosophila* potassium channel Shaw; Wei et al., 1990) utilizes alternative splicing of primary transcripts for expression of various potassium channels from one gene unit (Luneau et al., 1991a; Rettig et al., 1992; Yokoyama et al., 1989). The Raw spice variants that have been isolated thus far differ only in their carboxy terminal sequence. These sequence differences however do not give rise to expression of potassium channels with distinct electrophysiological or pharmacological properties (Luneau et al., 1991b; Rettig et al., 1992).

The prevailing type of potassium channels cloned thus far belongs to the delayed rectifier channel family. Rapidly inactivating, A-type potassium channels are not as abundant as the delayed rectifier type. Presently, only

one member from each of the four families of voltage-gated potassium channels has been found to express an inactivating , A-type channel (Stuhmer et al., 1989b; Baldwin et al., 1991; Pak et al., 1991a; Schroter et al., 1991). Non-inactivating potassium channels are considerably more abundant than rapidly inactivating ones (Rudy, 1988). Mouse homologues for all four *Drosophila* potassium channel genes have been cloned (Tempel et al., 1988; Mckinnon, 1989; Yokoyama et al., 1989; Grissmer et al. 1990; Pak et al., 1991a; Pak et al., 1991b). The mouse potassium channels have properties indistinguishable from their homologous rat potassium channels (Yokoyama et al., 1989; Grissmer et al. 1990; Baldwin et al., 1991; Pak et al., 1991a; Pak et al., 1991b).

## POTASSIUM CHANNEL DIVERSITY

Potassium channel diversity occurs not only at the DNA and RNA level but also at the protein subunit assembly level. Co-injection of two cRNAs of the Kv1 subfamily, each of which expresses different gating and pharmacological properties, led to the expression of single channels with mixed properties that was expected from the expression of both cRNAs

(Ruppersberg et al., 1990; Isacoff et al., 1990a). The interpretation was that heteromultimeric channels had been formed. In addition, whole-cell currents expressed by co-injection of cRNAs with different sensitivities toward blockade by external TEA were also similarly interpreted (Kavanaugh et al., 1991). Some of the most convincing data came from experiments in which tandem dimer constructs of two different cDNAs expressed single-channel currents with properties expected from heteromultimeric channels (Isacoff et al., 1990a). It was suggested that the assembly of heteromultimers might create potassium channels with novel properties as compared with corresponding homomultimers (Christie et al., 1990; Isacoff et al., 1990a; McCormack et al., 1990; Ruppersberg et al., 1990). This type of assembly would greatly enhance the possibilities for expressing potassium channels with more diverse properties than potassium channel proteins encoded in the genome. On the other hand, the unrestrained mixing of potassium channel subunits into heteromultimers would create problems for a given cell type. The cell would have difficulties regulating the number and kind of independent potassium channels formed. Fortunately, heteromultimer formation is not without constraints. Heteromultimers do not form from co-injection of cRNAs from members of different potassium channel families (Covarrubias et al., 1991; Li et al., 1992). These studies found that *Shaker*,

Shab, Shal, and Shaw subunits do not co-assemble in a detectable manner in the *Xenopus* oocyte expression system.

## FUNCTION FROM STRUCTURE: PREDICTIONS FROM AMINO ACID SEQUENCE

The structure and function of voltage-gated potassium channels and sodium channels has recently been reviewed (Catterall, 1991, Stuhmer, 1991; Mackinnon, 1991b; Miller, 1991; Lester, 1991; Catterall, 1992; Armstrong, 1992; Baldwin et al., 1992; Jan and Jan, 1992a; Miller, 1992a; Stuhmer and Parekh, 1992; Brown, 1993; Pongs, 1993a; Hoshi and Zagotta, 1993; Jan and Jan, 1994; Brown, 1994). In order to study protein function, one needs an understanding of protein structure. Without a crystal structure of the channel, the analysis of protein function has to be attacked by methods that interfere with or alter its function. This has led to the use of the relatively new technique of site-directed mutagenesis to study ion channels (Miller, 1989). This powerful technique has allowed the experimenter to infer protein structure from its altered function.

## VOLTAGE-DEPENDENT CHANNEL GATING

Voltage-dependent ion channels gate or open with membrane depolarization (Armstrong, 1974; Armstrong, 1992; Hille, 1992). Gating involves a voltage-dependent conformational transition of the closed channel into an activated state from which the channel can open in a voltage-independent manner. Voltage-dependent gating of ion channels implies that they sense the membrane potential by means of charges or voltage sensors intrinsic to the channel protein. Hodgkin and Huxley developed an empirical kinetic description to describe the opening of sodium and potassium channels (Hodgkin and Huxley, 1952d). The opening of potassium channels was supposed to be controlled by several independent gating particles bearing an electrical charge that makes their distribution in the membrane, voltage dependent. Although their original ideas and assumptions about gating were made without the knowledge of ion channels, the voltage-sensing gating particles have been shown to be integral components of the channel protein. In addition, movements of the charged voltage sensors have been directly measured as gating currents (Bezanilla et al., 1991; Stuhmer et al., 1991; Perozo et al., 1992; Perozo et al., 1993; Taglialatela and Stefani, 1993). Mutations in the S4 segment of voltage-dependent ion channels have

been shown to affect both voltage-sensing and gating currents (Stuhmer et al., 1989; Bezanilla et al., 1991; Papazian et al., 1991; Liman et al., 1991; Lopez et al., 1991; McCormack et al., 1991; Stuhmer et al., 1991; Logothetis et al., 1992; Schoppa et al., 1992; Logothetis et al., 1993). The S4 segment is the hallmark of voltage-sensitive ion channels. The S4 segment carries a number of positively charged amino acid side chains (lysine or arginine) generally spaced every three amino acids (Arg/Lys-X-X)n, where n may vary from 4 to 7. The intervening amino acids are generally hydrophobic residues, and have been shown to be intimately involved in voltage-dependent gating.

Mutations in the S4 segment of the *Shaker* potassium channel caused a shift of the midpoints of the voltage-dependent conductance curves (Papazian et al., 1991). From the slope of these curves, it was estimated that the charge carried by channel gating particles across the membrane was reduced by neutralizing some of the charged amino acids within the S4 segment. However, the presence or absence of positively charged amino acid side chains might not be correlated with gating charges. Substitutions of positively charged amino acids do not equally shift voltage-dependence or the slope of channel activation (Stuhmer et al., 1989; Papazian et al.,

1991). The kind of charged amino acid, arginine or lysine, may also influence the gating characteristics of the channel even though they both carry a positive charge (Papazian et al., 1991).

In order to better assess the role of the S4 domain in voltage sensing, I undertook a site-directed mutagenesis study determining the function of the interspersed hydrophobic residues in the S4 domain (Lopez et al., 1991). Conservative hydrophobic substitutions (generally leucine to alanine or valine) in the S4 segment of *Shaker* channels caused comparable or even larger shifts of the conductance-voltage curves along the voltage axis than the neutralization of basic amino acid residues (Papazian et al., 1991). Each of these mutations in the S4 sequence causes a large shift of the voltage-dependence curve of channel activation along the voltage axis, but does not alter the voltage-independent properties. Since similar mutations in the other proposed membrane spanning segments do not cause such shifts of the voltage-dependence curve, the S4 sequence appears unique with respect to its close involvement in the voltage dependence of channel activation. The effects of the leucine to alanine mutations in S4 are unusually large; the free energy difference ($\Delta G$) between the closed and the open (activated) state is altered in individual mutants by 4-20 kcal/mole, i.e. these S4 mutations

cause a change in the free energy difference (ΔΔG) of 1-5 kcal/mole per subunit, assuming that a potassium channel is a tetramer (Tempel et al., 1987; MacKinnon, 1991a) (Figure 3). By comparison, leucine to alanine mutations in other proteins destabilize the protein by only 0-3 kcal/mole by creating a cavity within the protein; the greatest destabilizing effect results when no adjustment of structures surrounding the cavity is found in the mutant protein (Eriksson et al., 1992). The leucine to alanine mutations also have an additional destabilizing effect of 2 kcal/mole, so that the mutant protein denatures more easily due to the difference in hydrophobicity between leucine and alanine (Eriksson et al., 1992). This latter component due to the hydrophobicity difference would not alter ΔG between the closed and open state of a channel, since the channel in either state is not expected to approach a denatured state and to expose a buried leucine. Thus, a difference in the free energy of the channel between the closed and the open state may be altered by the leucine to alanine mutations if such mutations have differential effects on the packing and stability of the channel in the closed and open states. If the only effects of these mutations were to destabilize the channel protein by creating a cavity, the maximum alteration of ΔG should be no more that 3 kcal/mole; the voltage-dependence curve would shift to the right if a larger cavity is created in the

channel at the open state, left if a larger cavity is created in the channel at the closed state. The observed largest shift of the voltage-dependence curve to the right corresponds to an alteration of the free energy difference between the closed state and the open state by 5 kcal/mole. This implies that substitution of alanine for leucine at position 375 (and, to a lesser extent, 361) releases some strain and thereby stabilizes the closed state; the unusually large alteration of the free energy difference could be accounted for by the sum of the effects of stabilizing the closed state and the effects of destabilizing the open state by creating a cavity. Thus these results indicate that the wild-type *Shaker* channel protein appears to be structurally constrained in a way that facilitates channel activation by depolarization. These results also suggest that the S4 sequence plays an important role in the conformational change that accompanies gating. A conformational change probably involves different parts of the channel that cooperatively alter their relative distribution within the electric field. The S4 sequence probably provides the interactions between other parts of the channel that are involved in the voltage-dependent conformational transition of the closed channel into an activated and open channel.

## INACTIVATION IN *SHAKER* POTASSIUM CHANNELS

The activation and inactivation gates were postulated to be completely independent in the formulation of Hodgkin and Huxley (Hodgkin and Huxley, 1952d). They theorized three gates for activation and one for inactivation for the sodium conductance. The idea of inactivation gates was further extended by studying artificially induced inactivation of potassium channels by TEA derivatives (Armstrong, 1966; Armstrong, 1968; Armstrong 1969; Armstrong, 1971) and by the knowledge that sodium channel inactivation could be removed by proteolytic enzymes (Armstrong et al., 1973; Rojas and Rudy, 1976). These results led Clay Armstrong to put forward the ball and chain model of channel inactivation (Armstrong and Bezanilla, 1977; Bezanilla and Armstrong, 1977). In the model, the ball is attached to the inner edge of the membrane by a strand of protein, which can be cut by the enzyme pronase. The ball has a receptor at the inner end of the channel that is protected by the activation gate. The normal sequence of events is that the activation gate opens following depolarization, allowing the channel to conduct until the ball diffuses into its receptor and inactivates the channel (Figure 4).

Macroscopic currents from a number of alternatively spliced *Shaker* variants expressed in *Xenopus* oocytes revealed that they mediated outward currents with varying inactivation kinetics (Timpe et al., 1988a; Timpe et al., 1988b; Iverson et al., 1988; Iverson et al., 1990). Both the alternative amino and carboxyl termini were originally implicated as these were the sites of differences between the different splice variants. Using a mutagenic approach, the localization of the ball and chain was determined for the *Shaker B* potassium channel. Using mutants with deletions at various points in the channel, it was discovered that the inactivation gate was at the amino terminus of the channel (Hoshi et al., 1990; Zagotta et al., 1990). In terms of balls and chains, it was found that the first 40 amino acids or so of the channel constituted the ball. Using synthetic peptides, they showed that when added to the internal medium, they caused inactivation in mutant channels that do not inactivate. The chain was composed of the next 60 amino acids in the channel. Shortening the chain speeded inactivation as though the ball had a shorter distance to travel. This type of inactivation was termed N-type for the amino terminus. The N-type of inactivation is also disrupted by mutations within the sequence between the S4 and S5 domains (Isacoff et al., 1991). These results indicate that the S4-S5 domain functions as a receptor for the inactivation ball. Another type of inactivation,

named C-type, was uncovered by both the same authors (Hoshi et al., 1991) and myself (G. Lopez, unpublished data). This was revealed by the observation that two splice variants of *Shaker, Shaker A* and *Shaker B*, had differing rates of slow inactivation. The main differences between the two channels was at the carboxyl termini. The amino acid differences began in the beginning part of the S6 domain. The S6 domains were identical except for two amino acids, an alanine and isoleucine. The *Shaker A* variant had almost negligible slow inactivation compared with the *Shaker B* variant. Mutating both these residue individually in *Shaker B*, I discovered that the valine to alanine mutation facilitates C-type inactivation.
The mutational analysis indicates that *Shaker* channels have two distinct options for inactivation.

## PERMEATION AND BLOCK IN VOLTAGE-GATED POTASSIUM CHANNELS

How does a membrane protein allow such a large number of ions to flow when it is open (10 million ions per second) and yet still be able to sense and select for only one type of ion? For example, the ionic permeability ratio of sodium versus potassium ($P_{Na}/P_K$) is less than 0.010 for

the delayed rectifier in myelinated frog nerve (Hille, 1973). In fact, potassium channels are among the most selective ion channels known (Hille, 1984). Most of what we know about the permeation pathway of potassium channels has come from experiments using analogs of the natural substrate potassium. Metal ions and alkylated ammonium derivatives have been used to probe the "mouths" and pores of various potassium channels. Many of these analogs bind to the mouth and inhibit potassium flux, while other analogs bind to the inside of the pore and impede current flow due to their high affinity. In addition, there are specific and high affinity ($K_D$ in the nM range) pore-blocking peptides which have been isolated from snake and scorpion toxins (Strong, 1990; Pongs, 1992).

Tetraethylammonium (TEA), the classical blocker of potassium channels, can block channels when applied intracellularly or extracellularly. If the TEA ion binds to a site in the channel that is part way across the membrane, the electrical potential energy of the ion will differ between the site and the bathing solution. The blocking ion will partition according to a Boltzmann distribution with the voltage-dependent affinity, $K_d(V)$, given by the equation:

$$K_d(V) = K_d(0) \exp (\delta ZFV / RT)$$

where $\delta$ is the fraction of the electrical field traversed by the blocker in moving from the inside solution into the blocking site (Woodhull, 1973). The electrical distance $\delta$, indicates the relative penetration of the blocker into the channel and so different blockers can be used to probe the relative size and shape of the entrance of the channel. There are several pieces of experimental evidence which indicate that TEA enters the pore to block conduction. First, internal TEA block is voltage dependent such that the blocking ion must traverse part way through the potential drop across the membrane ($\delta$ is approximately 0.15; French and Shoukimas, 1981; Spruce et al., 1987; Kirsch et al., 1991b; Taglialatela et al., 1991; Yellen et al., 1991). In addition, the smaller ammonium derivative tetramethylammonium (TMA) has a higher voltage-dependence indicating that it likely enters the channel further than TEA before blocking it (French and Shoukimas, 1985). Second, raising the extracellular concentration of potassium (or other permeant ions such as rubidium) while TEA is applied intracellularly, decreases the affinity of the TEA ion as if potassium or another permeant ion from the outer membrane is driving the TEA ion out of the channel (Armstrong, 1969; Armstrong, 1971; Taglialatela et al., 1991; Newland et al., 1992). This phenomenon localizes the action of TEA to the interior of the pore and demonstrates that more than one ion can be simultaneously

present and interact in the pore. Finally, TEA gains access to the internal binding site only when the voltage-dependent activation gate of the channel is open (Armstrong, 1966b). These studies laid the groundwork for future experiments using mutated potassium channels to localize the internal and external entrance and mouth of the channel and to identify at the amino acid level where TEA and other blockers bound.

## THE EXTERNAL MOUTH OF THE *SHAKER* POTASSIUM CHANNEL

Because of its pore blocking effect, the peptide charybdotoxin was used to identify parts of the *Shaker* potassium channel involved in toxin binding (MacKinnon and Miller, 1989b; MacKinnon et al., 1990). Using site directed-mutagenesis, it was found that certain residues in the proposed extracellular region H5 when mutated affected the channels sensitivity to externally applied charybdotoxin. They found that the negatively charged residues (glutamic acid and aspartic acid) at position 422 and 431 when neutralized (to glutamine and asparagine), decreased charybdotoxin binding affinity significantly. In addition, a threonine at position 449 when mutated to serine also decreased charybdotoxin binding. These results led the authors

to conclude that the region between the hydrophobic and presumed transmembrane domains S5 and S6 was close to and perhaps forms part of the toxin binding site and the external mouth of the ion channel. In another series of experiments, TEA was used to further characterize the external mouth of the *Shaker* potassium channel (MacKinnon and Yellen, 1990). Certain residues in the H5 region were found that affected the channel's sensitivity to external TEA. In particular, the threonine residue at position 449 when mutated to amino acids found in other potassium channels, yielded TEA sensitivities similar to those potassium channels. These studies helped localize the mouth of the *Shaker* potassium channel pore not only to a domain of the channel protein (the H5 domain was previously thought to be outside the membrane), but to individual amino acid residues within the channel protein. They also spurred on the hunt for the pore lining structure of the potassium channel protein.

## THE INTERNAL MOUTH AND PORE OF THE *SHAKER* POTASSIUM CHANNEL

In a follow up study, MacKinnon and Yellen used the same strategy

except this time searching for the internal TEA binding site (Yellen et al., 1991). TEA can block potassium channels from the extracellular and the intracellular side of the membrane. The difference between the two sites is that the binding of TEA to the internal site is sensitive to the transmembrane voltage in a fashion consistent with the partial penetration of TEA into the pore (Armstrong and Binstock, 1965; French and Shoukimas, 1981). Yellen and colleagues mutated highly conserved residues in the H5 domain and found that when they mutated the threonine at position 441, the internal TEA sensitivity decreased significantly while external TEA sensitivity was unaffected. From this result, the authors concluded that the H5 region must enter and cross the membrane twice and that 80% of the transmembrane electric potential falls across the eight amino acids between residues 441 and 449 (residue 449 was previously concluded to be on the outside of the membrane; Mackinnon and Miller, 1989b; Mackinnon et al., 1990). Recently, several of the cloned mammalian potassium channels were studied for their sensitivity to internal TEA (Taglialatela et al., 1991; Kirsch et al., 1991a; Kirsch et al., 1991b). Of the four channels examined, one of them NGK2, was less sensitive to TEA by a factor of more than 30-fold (Taglialatela et al., 1991). Surprisingly, the threonine residue at position 441 is 100% conserved in all cloned potassium channels (including several

adjacent residues; Figure 5), yet there is a wide range of internal TEA sensitivities. This implies that the internal TEA binding site is composed of several residues, possibly from different parts of the protein.

A more recent report provided additional evidence that the H5 region may contribute to the pore (Hartmann et al., 1991). The investigators took advantage of the fact that two of their cloned potassium channels differed significantly in several conductance properties (TEA sensitivity and single-channel conductance). They replaced a 21 amino acid region containing part of the H5 region from a channel with a very low sensitivity to internal TEA and large single-channel conductance (NGK2), into a channel with a high sensitivity to internal TEA and having a much lower single-channel conductance (DRK1). The resulting chimeric channel possessed the characteristic TEA profile and single-channel conductance of the donor NGK2 channel.

If the H5 region traverses the membrane twice as an anti-parallel $\beta$-strand as has been proposed (Yellen et al., 1991; Hartmann et al., 1991; Yool and Schwarz, 1991), this would place the methionine at position 440 at the end of one half of the $\beta$-strand. Additionally, the two proline residues

(positions 430 and 450) are likely to be at the extracellular end of the β-strands since the adjacent residues aspartic acid 431 and threonine 449 strongly influence the binding of external TEA (MacKinnon and Yellen, 1990). The sites influencing external TEA binding are necessarily extracellular and define the maximum length of the H5 region. If H5 forms an anti-parallel β-strand, then each residue would extend approximately 3.5 Å (Stryer, 1988) and one half of this strand could extend a maximum distance of only 27 Å through the membrane (the average thickness of a lipid membrane is approximately 40-50 Å; Stryer, 1988). If methionine 440 is at the furthest extent of the β-strand and if valine 443 forms part of the internal TEA binding site, then this would place valine 443 approximately 10.5 Å (3 * 3.5 Å) away from methionine 440 and put it closer to the extracellular side of the membrane. This would be inconsistent with the voltage-dependent calculation of $\delta$ (approximately 15%; assuming that the electrical potential decreases linearly across the thickness of the membrane) which is the fraction of the electrical distance traversed by the blocker TEA. It is therefore likely that either the TEA site consists of several residues or that other portions of the protein may contribute to the internal TEA binding site and form part of the pore lining structure.

Additional evidence for the H5 region forming part of the pore lining structure derives from experiments examining effects on ion selectivity from mutations in the H5 region (Yool and Schwarz, 1991). Using bi-ionic recording conditions to measure relative cation permeabilities, two mutations in H5 were found to increase ammonium permeability (ammonium normally has a very low permeability through potassium channels). To assess selectivity, currents can be recorded in bi-ionic conditions with potassium as the internal cation and the test ions $NH_4^+$, $Rb^+$, $K^+$, or $Na^+$ as the sole external monovalent cation. Bi-ionic recordings provide a measurement of cation permeability relative to potassium from the reversal potential (Hille, 1973). Relative permeability can be calculated from the change in reversal potential using the bi-ionic equation from Hille:

$$P_X/P_K = \exp [ (ZF/RT) (E_r) ],$$

where $P_X/P_K$ is the relative permeability to the test ion $X^+$ relative to potassium, and $E_r$ is the reversal potential. The two mutations that increased permeability to ammonium, phenylalanine 433 to serine, and threonine 441 to serine, also increased rubidium permeability with no detectable inward currents observed with external sodium even though sodium has a smaller ionic crystal radius. This implies that multiple barriers in the pore exploit steric hindrance and differences in both the ionic charge density and ion

dehydration energy in the permeation process. In fact, potassium channels are thought to have a series of potassium binding sites (Hille and Schwarz, 1978; Latorre and Miller, 1983; Begenisich and Smith, 1984) that, when unoccupied, have a high affinity for the permeant ion. When one of the sites is occupied, the affinity of neighboring sites decreases, presumably because of electrostatic repulsion between the bound ions in adjacent sites. The physiological concentration of the permeant ion ensures that neighboring sites are simultaneously occupied. Because of these proposed series of binding sites, and because of the high potassium selectivity, it is likely that one or several of them make up the selectivity filter of the channel. That is, there must be sites in the pore that can sense and select for only potassium ions.

Although there is experimental evidence suggesting that the H5 domain contributes to the pore structure, I propose that the adjacent presumed membrane spanning domain S6 also contributes to the pore lining structure. Given the proposed length of H5, it is not likely to traverse the entire width of the membrane and therefore may only contribute partially to the pore lining structure. The S6 domain is one of the most conserved domains in relation to the proposed membrane spanning domains S1, S2,

S3, and S5. In fact, S6 is as well conserved as H5 (Figure 6). It is likely therefore, that the H5 domain interacts with the S6 domain to form the pore lining structure of the *Shaker* voltage-gated potassium channel.

**FIGURES**

**FIGURE 1**

*a*

ShB1

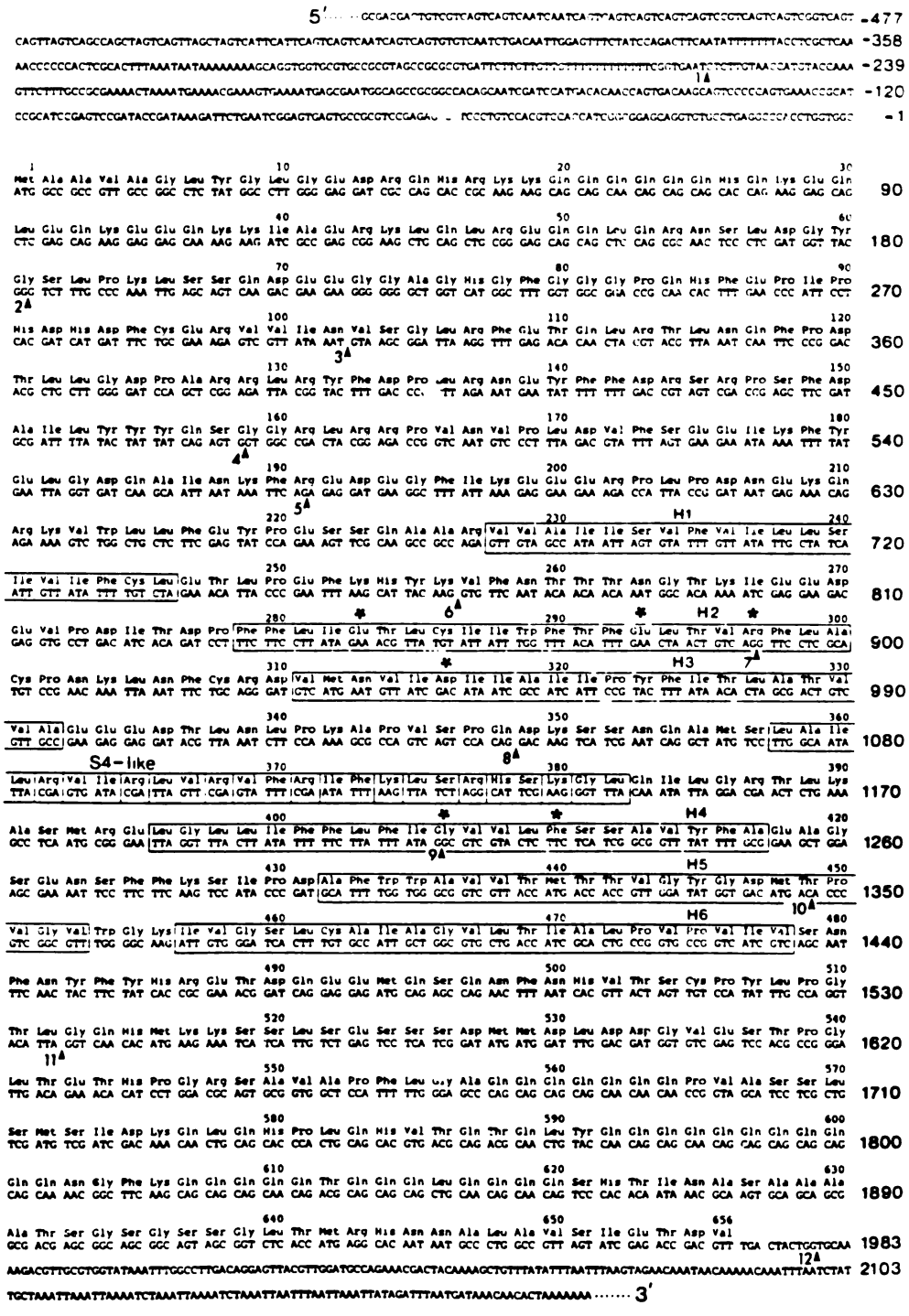


**FIGURE 1 Nucleotide and Amino-Acid Sequence of the *Shaker B* Voltage-Gated Potassium Channel.**

Nucleotide and predicted amino acid sequence of *Shaker B*. The complete sequence is given with nucleotides numbered on the right. Segments that may span the membrane are boxed and labelled: H1-H6 are hydrophobic segments (Scwharz et al., 1988).

**FIGURE 2**

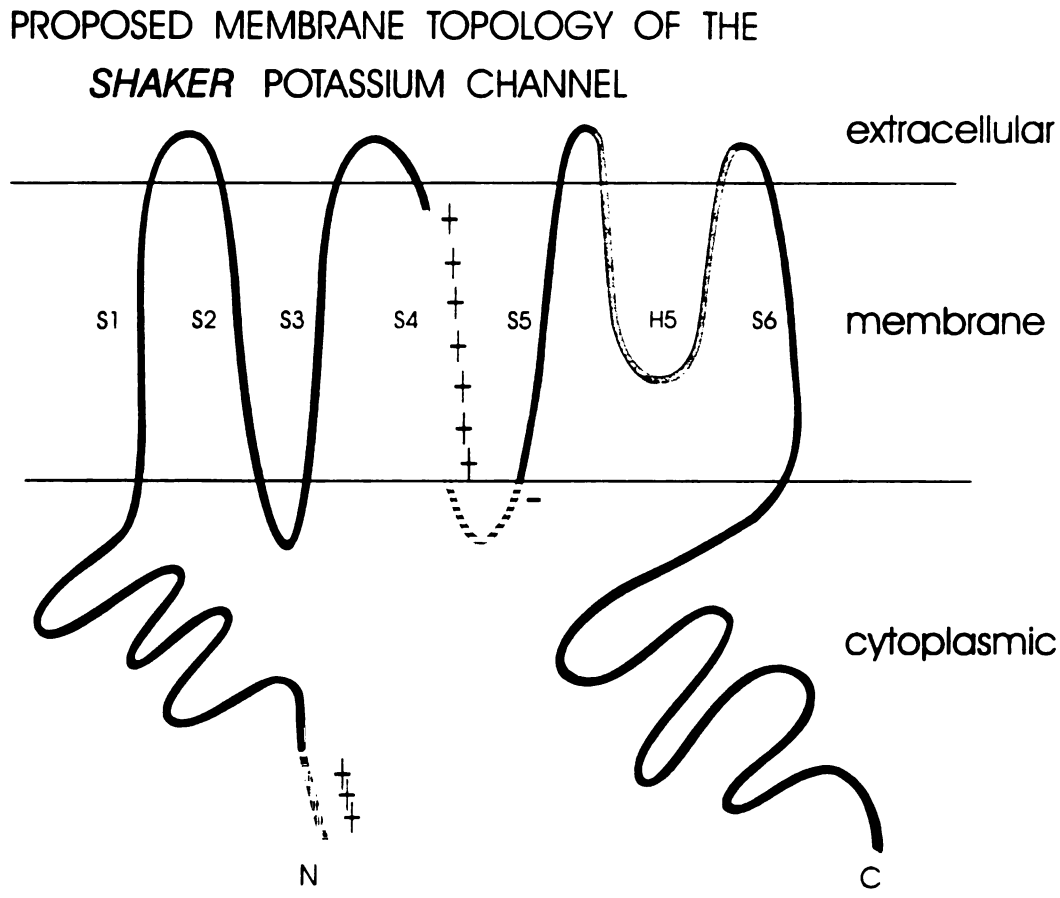
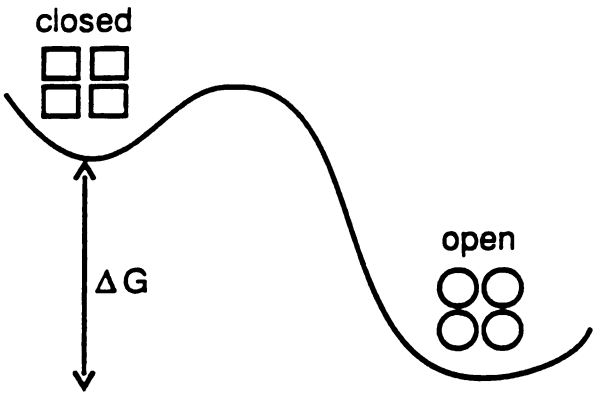

PROPOSED MEMBRANE TOPOLOGY OF THE
*SHAKER* POTASSIUM CHANNEL
extracellular
membrane
cytoplasmic
S1
S2
S3
S4
S5
H5
S6
N
C

**FIGURE 2 Proposed Membrane Topology of the *Shaker B* Voltage-Gated Potassium Channel.**

Cartoon picture of the proposed topology of the potassium channel. Hydrophobic transmembrane domains are labelled as S1 through S6. The H5 domain has been theorized to enter and leave the membrane. The S4 domain is shown with 7 positively charged amino acid residues. The amino terminal domain involved in fast inactivation is shown with three positively charged amino acids. The S4-S5 domain which has been shown to be part of the receptor for the inactivation gate is shown with a negatively charged amino acid (glutamate 395).

**FIGURE 3**

A Simple Two-State Model for Voltage-Dependent Channel Gating



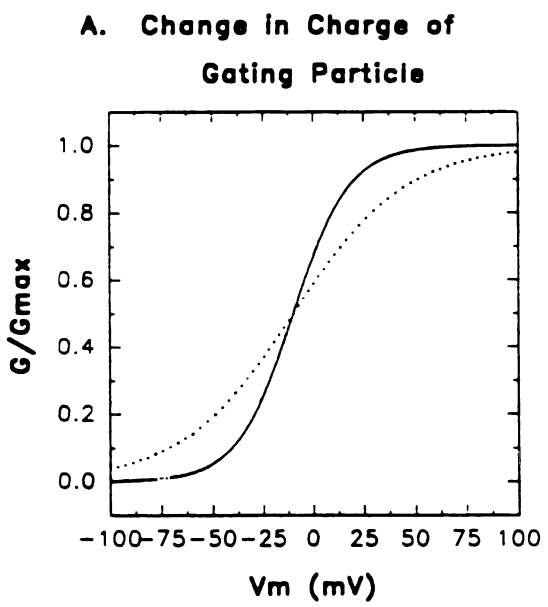

1. $Vm = 0$

$$\frac{open}{closed} = \exp(\Delta G/RT)$$

2. $Vm = \Delta V$

$$\frac{open}{closed} = \exp(-\Delta G/RT + zF\Delta V/RT)$$

Rearrange: $$f(open) = \frac{1}{1 + \exp(\Delta G/RT - zF\Delta V/RT)}$$



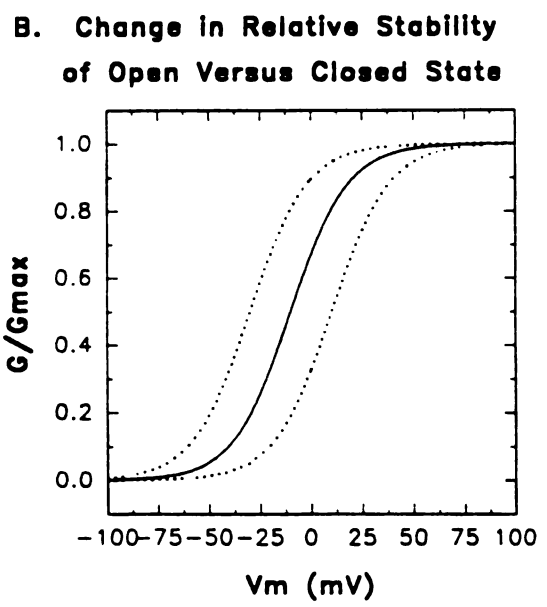



**FIGURE 3 A Simple Two-State Model for Voltage-Dependent Channel Gating**

Voltage-dependence of channel activation is illustrated by a simple two-state model. The fraction of channels that are open (f[open]) at membrane potential Vm depends on the charge of the intrinsic gating particle (z) and the difference in free energy between the open and closed state (ΔG). A change in ΔG by a mutation will shift the normalized conductance-voltage curve along the voltage axis. G = conductance; Vm = membrane potential.

**FIGURE 4**

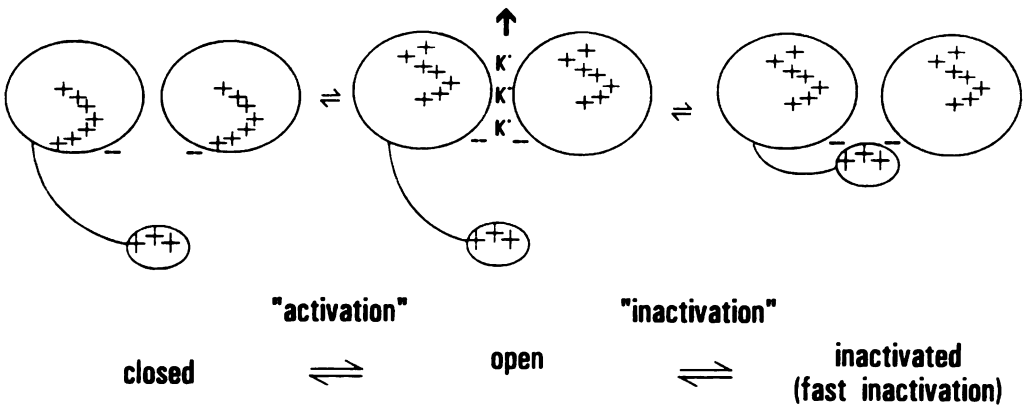

K+
K+
K+
"activation"
"inactivation"
closed
open
inactivated
(fast inactivation)

**FIGURE 4 A Simplified Scheme of Potassium Channel Gating and Permeation**

A cartoon picture representing the potassium channel moving from the closed state into the activated state during a membrane depolarization. The channel subsequently opens and allows the outflux of intracellular potassium ions to flow through the pore. The channel will then undergo fast inactivation, as is the case with *Shaker B* channels, with the amino terminus occluding the inner mouth of the channel.

**FIGURE 5**

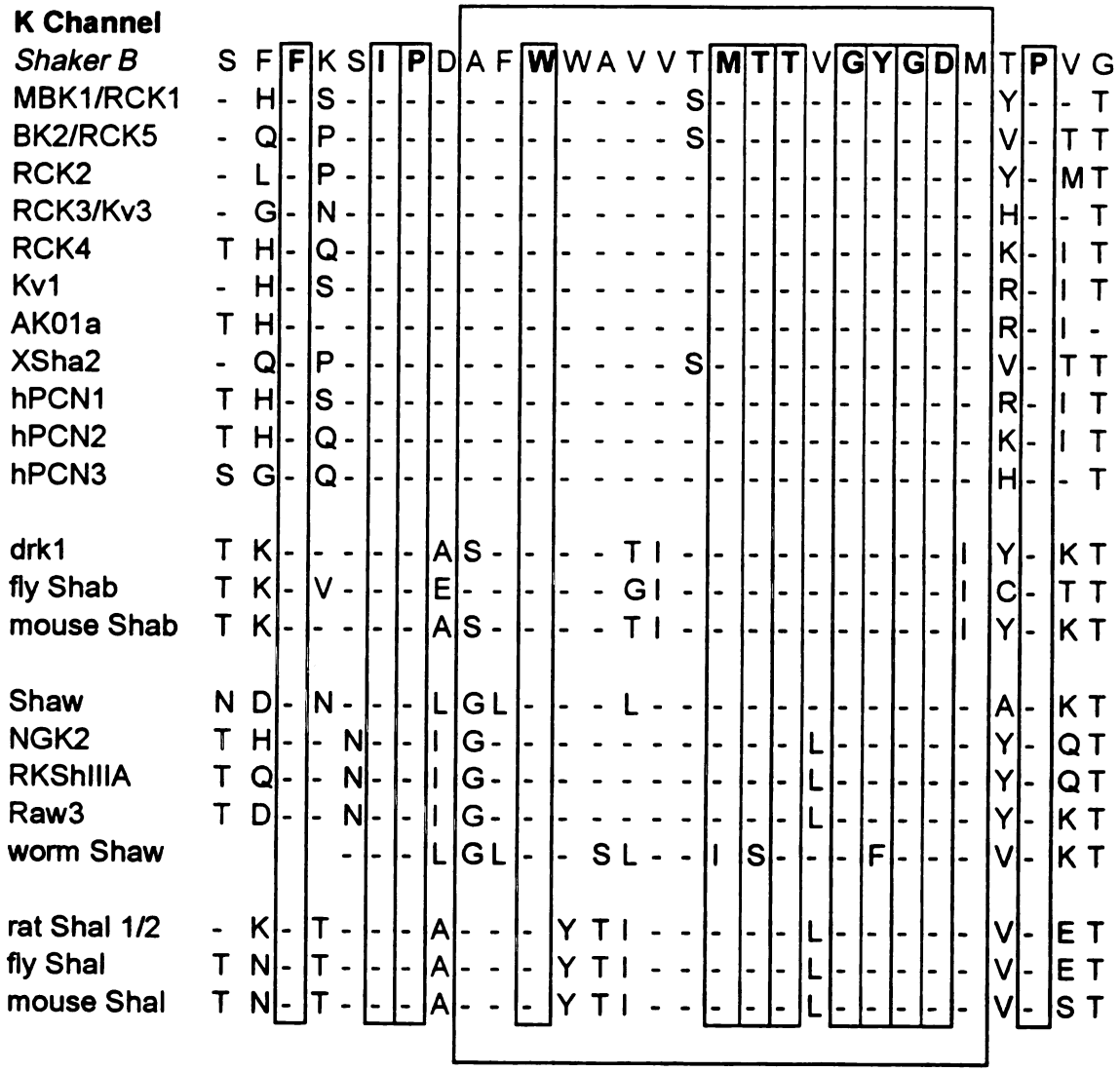

H 5
K Channel
Shaker B S F F K S I P D A F W W A V V T M T T V G Y G D M T P V G
MBK1/RCK1 - H - S - - - - - - - - - - - S - - - - - - - - Y - - T
BK2/RCK5 - Q - P - - - - - - - - - - - S - - - - - - - - V - T T
RCK2 - L - P - - - - - - - - - - - - - - - - - - - - Y - M T
RCK3/Kv3 - G - N - - - - - - - - - - - - - - - - - - - - H - - T
RCK4 T H - Q - - - - - - - - - - - - - - - - - - - - K - I T
Kv1 - H - S - - - - - - - - - - - - - - - - - - - - R - I T
AK01a T H - - - - - - - - - - - - - - - - - - - - - R - I -
XSha2 - Q - P - - - - - - - - - - S - - - - - - - - V - T T
hPCN1 T H - S - - - - - - - - - - - - - - - - - - - R - I T
hPCN2 T H - Q - - - - - - - - - - - - - - - - - - - K - I T
hPCN3 S G - Q - - - - - - - - - - - - - - - - - - - H - - T
drk1 T K - - - - - A S - - - - T I - - - - - - - - I Y - K T
fly Shab T K - V - - - E - - - - - G I - - - - - - - - I C - T T
mouse Shab T K - - - - - A S - - - - T I - - - - - - - - I Y - K T
Shaw N D - N - - - L G L - - - L - - - - - - - - - A - K T
NGK2 T H - - N - - I G - - - - - - - - - L - - - - Y - Q T
RKShIIIA T Q - - N - - I G - - - - - - - - - L - - - - Y - Q T
Raw3 T D - - N - - I G - - - - - - - - - L - - - - Y - K T
worm Shaw - - - L G L - - S L - - I S - - F - - - V - K T
rat Shal 1/2 - K - T - - - A - - - Y T I - - - - - L - - - - V - E T
fly Shal T N - T - - - A - - - Y T I - - - - - L - - - - V - E T
mouse Shal T N - T - - - A - - - Y T I - - - - - L - - - - V - S T

**FIGURE 5 Amino Acid Alignment of the H5 Domain from Various Cloned Voltage-Gated Potassium Channels from All Three Subfamilies**

Amino acids are aligned with the *Shaker B* potassium channel. Amino acids which are 100% conserved are in bold type and boxed. The larger box represents the entire H5 domain. Single letter code for the amino acids are: S, serine; F, phenylalanine; K, lysine; I, isoleucine; P, proline; D, aspartic acid; A, alanine; W, tryptophan; V, valine; T, threonine; M, methionine; G, glycine; Y, tyrosine; C, cysteine, H, histidine; L, leucine.

**FIGURE 6**

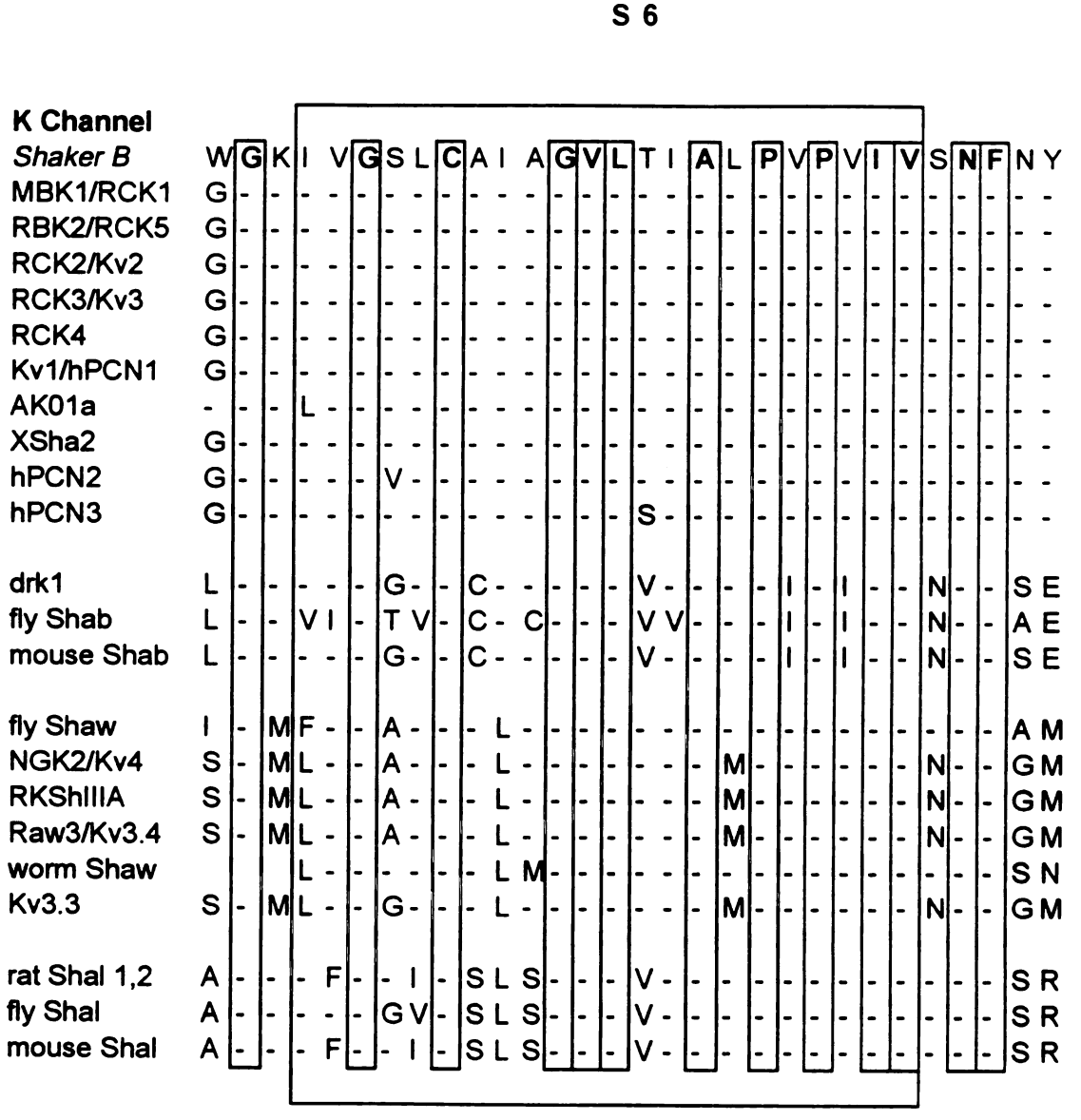

S 6
K Channel
Shaker B W G K I V G S L C A I A G V L T I A L P V P V I V S N F N Y
MBK1/RCK1 G - - - - - - - - - - - - - - - - - - - - - - - - - - - -
RBK2/RCK5 G - - - - - - - - - - - - - - - - - - - - - - - - - - - -
RCK2/Kv2 G - - - - - - - - - - - - - - - - - - - - - - - - - - - -
RCK3/Kv3 G - - - - - - - - - - - - - - - - - - - - - - - - - - - -
RCK4 G - - - - - - - - - - - - - - - - - - - - - - - - - - - -
Kv1/hPCN1 G - - - - - - - - - - - - - - - - - - - - - - - - - - - -
AK01a - - - L - - - - - - - - - - - - - - - - - - - - - - - -
XSha2 G - - - - - - - - - - - - - - - - - - - - - - - - - - - -
hPCN2 G - - - - V - - - - - - - - - - - - - - - - - - - - - - -
hPCN3 G - - - - - - - - - - - - S - - - - - - - - - - - - - -
drk1 L - - - - - G - - C - - - - V - - - - I - I - - N - - S E
fly Shab L - - V I - T V - C - C - - - V V - - - I - I - - N - - A E
mouse Shab L - - - - - G - - C - - - - V - - - - I - I - - N - - S E
fly Shaw I - M F - - A - - - L - - - - - - - - - - - - - - - A M
NGK2/Kv4 S - M L - - A - - - L - - - - - - - M - - - - - N - - G M
RKShIIIA S - M L - - A - - - L - - - - - - - M - - - - - N - - G M
Raw3/Kv3.4 S - M L - - A - - - L - - - - - - - M - - - - - N - - G M
worm Shaw L - - - - - - L M - - - - - - - - - - - - - - - - S N
Kv3.3 S - M L - - G - - - L - - - - - - - M - - - - - N - - G M
rat Shal 1,2 A - - - F - - I - S L S - - - V - - - - - - - - - - - S R
fly Shal A - - - - - G V - S L S - - - V - - - - - - - - - - - S R
mouse Shal A - - - F - - I - S L S - - - V - - - - - - - - - - - S R

**FIGURE 6 Amino Acid Alignment of the S6 Domain of Various Cloned Voltage-Gated Potassium Channels from All Four Subfamilies**

Amino acids are aligned with the *Shaker B* potassium channel. Amino acids which are 100% conserved are in bold type and boxed. The larger box represents the entire S6 domain. Single letter code for the amino acids are: S, serine; I, isoleucine; A, alanine; W, tryptophan; V, valine; T, threonine; M, methionine; G, glycine; Y, tyrosine; K, lysine; L, leucine; C, cysteine; P, proline; N, asparagine; F, phenylalanine; R, arginine; E, glutamate.

# CHAPTER II

## Hydrophobic Substitution Mutations in the S4 Sequence Alter Voltage-Dependent Gating in *Shaker* Potassium Channels

**ABSTRACT**

Voltage-activated sodium, calcium, and potassium channels contain a common motif, the S4 sequence characterized by a basic residue at every third position interspersed mainly with hydrophobic residues. The S4 sequence is proposed to function as the voltage sensor and to move in response to membrane depolarization, triggering conformational changes that open the channel. This hypothesis has been tested in previous studies, which revealed that mutations of the S4 basic residues often shift the curve of voltage-dependence of activation along the voltage axis. We find that comparable or larger shifts are caused by conservative substitutions of hydrophobic residues in the S4 sequence of the *Shaker* potassium channel. We suggest that the S4 structure plays an essential role in determining the relative stabilities of the closed and open states of the channel.

## INTRODUCTION

Voltage-dependent activation of sodium, calcium, and potassium channels is essential for membrane excitability and synaptic transmission, and poses an interesting question regarding protein structure and function. For a voltage-sensitive ion channel to detect changes of the membrane potential, the protein should have charges or dipoles in the hydrophobic portion of the membrane (Hille, 1984). These charged residues are probably displaced by the electrostatic force of membrane depolarization because a net outward movement of positive charges within the channel molecule (the gating current) has been observed during channel activation, leading to the translocation of the equivalent of about 4-8 positive charges across the membrane (the gating charge) (Armstrong and Bezanilla, 1974; Bezanilla et al., 1982; Hille, 1984; Zagotta and Aldrich, 1990a). It has been proposed that the S4 sequence, found in each known potassium channel polypeptide (Tempel et al, 1987; Tempel et al., 1988; Frech et al., 1989; McKinnon, 1989; Stühmer et al., 1989b; Grupe et al., 1990; McCormack et al., 1990; Ribera, 1990; Swanson et al., 1990; Wei et al., 1990; Pfaffinger et al., 1991) and in each of the four internally homologous domains of the alpha

subunit of sodium channels (Noda et al., 1984) and calcium channels (Tanabe et al., 1987), spans the membrane so that its basic residues are in the hydrophobic membrane interior and function as part of the voltage sensor (Greenblatt et al., 1985; Kosower, 1985; Catterall, 1986; Guy and Seetharamulu, 1986, Noda et al., 1986). These basic residues are hypothesized to move in response to membrane depolarization toward the extracellular surface, thereby leading to conformational changes that open the channel.

The hypothesis that the S4 sequence functions as the voltage sensor predicts that a reduction of the net positive charges in the S4 sequence should reduce the sensitivity of the channel to voltage. Such effects have been observed by mutating some of the S4 basic residues in the rat brain II sodium channel (Stühmer et al., 1989a) and the *Shaker* potassium channel (Papazian et al., 1991). However, there is a wide range of mutant phenotypes depending on the type of mutations and the specific basic residues mutated. For example, the most frequently observed phenotype is a shift of the voltage-dependence curves along the voltage axis when a basic residue is replaced with either a different basic residue or the neutral amino acid glutamine (Papazian et al., 1991). These mutant phenotypes cannot be explained simply by considering the electrostatic interactions

between the membrane electric field and the S4 basic residues, indicating that other structural considerations are important. Because the dielectric constant is likely to be small within the hydrophobic interior of a protein (Matthew, 1985; Gilson and Honig, 1986) and a charged residue may have significant electrostatic interactions with residues over distances as large as 10-20 Å (Russell et al., 1987; Gilson and Honig, 1988), mutations of the S4 basic residues have the potential of altering the long-range electrostatic interactions, and are therefore less than ideal for the purpose of assessing the involvement of the S4 sequence as a structural element in voltage-dependent activation.

To examine the potential structural involvement of the S4 sequence in channel activation, we have made conservative mutations of hydrophobic residues with non-ionizable, less polarizable side chains, because they are expected to have mainly short-range hydrophobic interactions with residues that are in close contact with them (Bondi, 1964; Abraham and Leo, 1987). To control for the possibility that such mutations might alter the packing of the channel protein to an extent that would grossly rearrange protein folding, we characterized several different properties of the channel, including macroscopic inactivation kinetics, potassium selectivity, as well as the voltage-dependence of activation and inactivation, by carrying out two-

electrode voltage-clamp studies of *Xenopus* oocytes injected with RNA transcribed *in vitro*. Similar mutations of hydrophobic residues in the S4 sequence and in other proposed membrane spanning regions of the *Shaker* B (ShB) alternative splice variant (Schwarz et al., 1988) were compared in order to assess the role of the S4 sequence in voltage-dependent activation.

In most experiments, we substituted a single leucine residue with alanine because such mutations preserve the hydrophobic nature of the side chain; the side chains of both leucine and alanine consist of only methyl groups, which are less likely to form induced dipoles than other side chains containing hydroxyl groups, sulfur, or aromatic rings. Furthermore, the alanine side chain is expected to occupy the position of the $\beta$ carbon of leucine, leading to a minimal perturbation of the packing of the protein (Ponder and Richards, 1987). By contrast, substitution of leucine with other hydrophobic residues such as methionine or valine would introduce additional methyl groups at locations that are not expected to harbor any carbon atoms of the leucine side chain in the wild-type protein, because the conformations, including the chi angles ("rotamers") of side chains in proteins are those of the lowest energy for the respective amino acids (Janin et al., 1978; Ponder and Richards, 1987). We chose to mutate only those hydrophobic residues that are highly conserved among the *Shaker* subfamily

of voltage-activated potassium channels from different species as these residues are more likely to interact with other parts of the channel protein rather than with lipids in the membrane. In the photosynthetic reaction center, a membrane protein of known structure, residues that face the lipid bilayer are found to be hydrophobic but less conserved than residues that interact with other membrane spanning segments of the protein (Yeates et al., 1987; Komiya et al., 1988).

We report here that several conservative substitutions of highly conserved hydrophobic residues in the S4 but not in the other proposed membrane spanning segments have large and specific effects on the voltage-dependence of channel activation. These results suggest that residue-residue interactions change during activation for all of the residues mutated in the S4 sequence whereas this does not appear to be the case for the other transmembrane segments. The structural and mechanistic implications of our findings are discussed in light of what is known of protein structure and channel function.

## METHODS

### Site Directed Mutagenesis

Oligonucleotide-directed mutagenesis of a uracil-substitute single stranded DNA template (Kunkel et al., 1987) was used to make mutations in the Shaker B clone (Schwarz et al., 1988). Template DNA was prepared from the dut$^-$ ung$^-$ bacterial strain RZ1032 (Kunkel et al., 1987). Oligonucleotides 20 to 26 bases in length were used to introduce one, two or three base pair mismatches. A phosphorylated oligonucleotide was annealed to the template DNA at an approximate molar ratio of 10:1 and was extended using the enzyme Sequenase (USB) in the presence of 1 unit of T4 DNA ligase, 1 mM rATP, 0.25 mM each of dATP, dCTP, dGTP and dTTP in a buffer containing 50 mM Tris (pH 7.5), 10 mM $MgCl_2$ and 10 mM dithiothreitol. After transformation into the BSJ-72 strain of *E. coli* bacteria, mutant clones were identified either by colony screening using radioactively labelled mutagenic oligonucleotide or by restriction mapping, for mutations that introduce a change in the restriction map. The mutant clones were then sequenced using either single stranded or double stranded DNA sequencing (Sanger et al., 1977; Mierendorf and Pfeffer, 1987). In order to help insure

that no second site mutations were created in the procedure, the following controls were done: (1) all of the mutant clones were sequenced in a region of 100 to 200 bases on either side of the mutation, (2) at least two independent clones were isolated for each mutant and were found to have the same electrophysiological phenotype.

### *In Vitro* Transcription

Once the mutant clones were identified by sequencing, the template DNA (approx. 2 $\mu$g) was linearized with the restriction enzyme Hind III. RNA was transcribed using 0.5 mM each of rATP, rCTP, rGTP, rUTP and the cap analog diguanosine triphosphate (Pharmacia) in a standard transcription buffer containing 40 mM Tris (pH 8.0), 8 mM $MgCl_2$, 2 mM spermidine, 50 mM NaCl and 75 mM dithiothreitol. 50 units of T3 RNA polymerase was used in the presence of 80 units of RNase inhibitor (Promega) for 2 hours at 37°C. The template DNA was then digested using 2 units of RNase-free DNase (Promega) for 30 minutes at 37°C. The RNA was purified using phenol/chloroform extractions followed by ethanol precipitation. To confirm the size and integrity of the RNA produced, denaturing gel electrophoresis was performed using one tenth of the transcription reaction and the migration compared to an RNA size ladder (BRL). The RNA was denatured in a solution of 50% formamide and 2.2 M formaldehyde and heated at 65°C

for 2 minutes prior to loading onto a 0.7% agarose, 6.7% formaldehyde gel. The RNA was stored at -70°C in sterile water.

**Oocyte Injection**

Adult female *Xenopus laevis* were anesthetized by immersion in ice water for 1 hour followed by surgical removal of several ovarian lobes. Oocytes were manually separated from the ovarian connective tissue prior to injection. Stage V and VI oocytes (Dumont, 1972) were injected with approximately 50 nl of RNA (1-2 mg/ml) and incubated in modified Barth's saline (MBS) containing 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 10 mM HEPES (pH 7.5), 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, and 0.41 mM $CaCl_2$ (Gurdon and Wickens, 1983) at 18°C. Twenty four hours following injection, oocytes were incubated in MBS with 2 mg/ml collagenase (cls 3, Worthington) for 2 hours with gentle shaking. This treatment helps remove the overlying connective tissues and follicular cells. Oocytes were incubated in MBS supplemented with 100 U/ml penicillin, 100 $\mu$g/ml streptomycin and 50 $\mu$g/ml gentamicin for 2-4 days before recording.

**Electrophysiological Recordings**

Ionic currents were recorded using a standard two-microelectrode voltage clamp circuit. Data acquisition and analysis were performed on a

80386 based micro-computer using the program pCLAMP and an A/D converter (Axon Instruments). Voltage and current micro-electrodes were filled with 3M KCl and had resistances of less than 2MΩ. Series resistance, approximated at 500 ohms (Papazian et al., 1991), was compensated for. Current signals were samples at 5 kHz and low-pass filtered at 1 kHz with an 8-pole Bessel filter. Leakage currents were subtracted by adding responses to scaled 20 mV hyperpolarizing pulses (P/4 method, Bezanilla and Armstrong, 1977). The *Shaker* potassium channel has an approximately linear open channel current-voltage relation between +30 and +80 mV (MacKinnon and Yellen, 1990) and between +20 and +120 mV (E. Isacoff, personal communication). The reversal potential at 1 mM potassium (-100 mV) was derived by linear regression on a semi-logarithmic plot using the reversal potential measurements in 20 and 40 mM external potassium because the tail currents were too small and the kinetics too fast in 1 mM potassium for accurate measurements. The conductance was calculated by dividing the leak subtracted peak current by the driving force. The theoretical curves fitting the conductance-voltage relations were determined using an iterative non-linear regression fitting procedure to the Boltzmann equation:

$$G / G_{max} = 1 / [ 1 + \exp (( V_{0.5} - V_m ) / b) ]$$

where $V_m$ is the membrane potential. From this fit, a slope parameter (b) and the midpoint potential ($V_{0.5}$) are obtained. Similarly, the form of the Boltzmann equation used to fit the prepulse-inactivation curve is:

$$I_p / I_{p,max} = 1 / [ 1 + \exp (( V_m - V_{0.5} ) / b) ]$$

where $V_m$ is the membrane potential, $V_{0.5}$ is the midpoint potential, and b is the slope parameter. All experiments were carried out at 11 ± 1 °C.

## RESULTS

### Voltage-Dependence of Activation is Affected by S4 Mutations

Each of the proposed membrane spanning segments of the *Shaker* potassium channel can be aligned with the analogous regions of other known potassium channel sequences. At least one highly conserved leucine residue in each segment was mutated to alanine (Figure 1). (The H5 sequence, which has been modeled to extend into the membrane (Guy and Conti, 1990), does not contain any leucine residues and is therefore not included in this study.) The S4 sequence has five leucine residues, four of which were mutated to alanine and one was mutated to valine. In addition, an alanine residue in the S4 sequence was mutated to leucine (Figure 2). The voltage-dependence of activation was determined by measuring the peak current elicited during a 500 msec depolarizing pulse to various potentials, as a function of voltage. Conductance was calculated by dividing the leak subtracted current by the driving force for potassium across the membrane using the extrapolated reversal potential of -100 mV (Table 2, see Methods). By plotting the conductance values as a function of membrane

potential and fitting with the Boltzmann equation, the midpoints and slope of the derived conductance-voltage relation were quantified and mean values for several oocytes analyzed individually were obtained (Table 1). Each mutation of a hydrophobic residue in the S4 sequence gave rise to a large shift along the voltage axis in channel activation (the conductance-voltage curve) (Figure 3B). Two of the six S4 mutants gave a hyperpolarizing shift in the conductance-voltage curve. The remaining four S4 mutations gave a depolarizing shift with the most dramatic being a shift of approximately 86 mV (L375A) (Table 1). (The mutations are denoted by the amino acid present in *Shaker* B (Schwarz et al., 1988) [first letter] at the position indicated by the number, followed by the amino acid present in the mutant [last letter]). There was no correlation between the position of the mutation in the S4 sequence and the direction of the shift in the conductance-voltage curve. Although there was a dramatic shift of the midpoint in the S4 mutants, the slopes of the curves were not altered significantly (Table 1) as would be expected for mutations not affecting the net charge of the S4.

In contrast to the S4 mutations, substituting leucine with alanine in any of the other five proposed membrane spanning segments had very small effects on the voltage-dependent properties of channel gating (Figure 3A, Table 1). Therefore, the S4 sequence appeared to be much more centrally

involved in voltage-dependent gating than the other five proposed membrane spanning segments. As described below, the effects of the S4 hydrophobic substitution mutations on the voltage-independent properties were mild, or not significant, indicating that these mutations did not grossly alter channel structure. Thus, it appears that slight alterations of the hydrophobic interactions between the S4 sequence and its surrounding were sufficient to cause a substantial shift in the voltage-dependence of channel activation.



**The S4 Mutations Affect the Voltage-Dependence of Macroscopic Inactivation**

The voltage-dependence of inactivation was determined by applying 500 msec depolarizing prepulses to a series of different potentials and then measuring the peak current evoked in response to a subsequent test pulse to a fixed potential, which allows maximal activation of channels. The midpoints and slope parameters determined from a Boltzmann fit to the data show minimal changes in the non-S4 mutant channels (Figure 3C, Table 1). In contrast, all of the S4 mutants showed a shift in the midpoints (Figure 3D, Table 1). As was found for the voltage-dependence of activation, two

S4 mutations gave a hyperpolarizing shift whereas the other four gave a depolarizing shift.

Very similar effects were found for the hydrophobic substitution mutations on the midpoints of the activation and prepulse inactivation curves (Figure 4), as has been observed when the basic residues of the *Shaker* potassium channel S4 sequence were mutated (Papazian et al., 1991). These results are consistent with previous single channel analysis (Zagotta et al., 1989b; Zagotta and Aldrich, 1990a) which shows that the wild-type Shaker potassium channel tends to open before it inactivates and that the process of inactivation is voltage-independent. Thus, the apparent voltage-dependence of inactivation of the macroscopic current recorded from a cell merely reflects the voltage-dependence of channel activation which precedes inactivation.

No appreciable changes in the slopes were detected in the non-S4 mutants or in five of the S4 mutants. The only exception is the mutant L366A, which showed an increase in the slope of the voltage-dependence of macroscopic inactivation but not in the slope of the voltage-dependence of activation (Table 1). Given the assumptions and technical limitations inherent in these slope measurements, as discussed in a previous paper

(Papazian et al., 1991), further experiments are necessary for a better understanding of the quantitative difference in the effects of a mutation on the slopes of the voltage-dependence curves. A similar effect has been observed in the R377K mutation (Papazian et al., 1991), which is also not expected to alter the number of charges in the S4 sequence. It is conceivable that in both cases the mutations affect the slope of the voltage-dependence curve by changing the cooperativity of channel activation (Papazian et al., 1991).

**Voltage-Independent Properties are Minimally Affected by the S4 Mutations**

In order to ensure that these hydrophobic substitutions in the S4 sequence did not affect channel protein folding in a global manner, we measured other channel properties that are independent of membrane potential: the kinetic properties of macroscopic inactivation and the potassium selectivity as indicated by the reversal potential.

The kinetics of fast inactivation of the macroscopic current was determined by fitting an exponential function to the decay phase of the current evoked by a 500 msec depolarizing pulse. It has been shown for the

wild-type Shaker potassium channel that the latency before first channel opening is greatly reduced at highly depolarized membrane potentials, so that most of the channels have opened as the macroscopic current reaches its peak value, and the macroscopic inactivation rate reflects largely the kinetics of the voltage-independent process of inactivation (Zagotta et al., 1989b; Zagotta and Aldrich, 1990a). Two time constants were used to fit the decay phase of the macroscopic currents. The first time constant, as given in Table 2, gave an accurate measurement of the fast component of macroscopic inactivation. The second component was in the range of 1000 msec and could not be assessed adequately using a 500 msec depolarizing pulse. Longer pulses were not used because at the large depolarizations necessary for the activation of some of the mutant channels, the slowly activating endogenous oocyte currents contaminated the current traces. Except for mild effects observed in L366A and L382V, the S4 mutations did not affect the time course of macroscopic inactivation (Table 2). Part of the mild effect of L366A could be accounted for by the large hyperpolarizing shift of its voltage-dependence curve of activation, since the mutant channels are likely to activate with much shorter latency at the same depolarized potential. As to the mutations which shifted the midpoints of the conductance-voltage curves in the depolarized direction, although the rate of inactivation appeared slow relative to the wild-type *Shaker* B at the

same membrane potential (Figure 2), the kinetics of fast inactivation were similar at comparable levels on the conductance-voltage curve (i.e. at or approaching Gmax).

The potassium selectivity of the wild-type and mutant channels was compared by measuring the reversal potential at 20 mM and 40 mM external potassium concentration (Table 2), which was determined by evoking tail currents in 5 mV increments in the voltage range that encompasses the reversal potential. With the exception of L375A, which only began to activate at +40 mV and gave no detectable tail currents for a reliable measure of the reversal potential, the S4 mutations did not alter the reversal potential or its dependence on potassium concentration (Table 2).

In summary, although each of the six hydrophobic substitution mutations in the S4 sequence caused large shifts of the voltage-dependence curves of activation and macroscopic inactivation, they did not alter the reversal potentials and had at most a mild effect on macroscopic inactivation kinetics. These S4 mutations specifically affect the voltage-dependent properties. Therefore, the mutant phenotypes are not likely to arise from global alterations of channel protein folding, rather, they are likely to be due to perturbations of the local interaction between the hydrophobic side chain

of an S4 hydrophobic residue (leucine, in five of the six mutants) and its immediate surrounding in the closed and/or the open configuration of the channel.

## DISCUSSION

By making conservative substitutions of highly conserved hydrophobic residues in the S4 sequence and in the other five proposed membrane spanning segments, we have found that mutations in the S4 sequence produced substantial and specific alterations of the voltage-dependent properties of the *Shaker* potassium channel. The magnitudes of the effects of each of these S4 mutations were large and comparable with those due to mutations of the basic residues in the same S4 sequence (Papazian et al., 1991), whereas similar hydrophobic substitution mutations in five other proposed membrane spanning segments did not produce such effects. These findings have three main implications: (i) Functionally, the S4 sequence appears to be intimately involved in voltage-dependent activation of the channel, (ii) structurally, the S4 sequence is likely to be buried within the channel protein so that most of its hydrophobic residues interact closely with other parts of the channel and (iii) mechanistically, the hydrophobic interactions between the S4 sequence and its immediate surrounding are likely to be different in the open and closed states of the channel, so that

even the most conservative mutations of the S4 hydrophobic residues severely alter the relative stabilities of the open and closed states.

**The S4 Sequence as a Structural Element Plays an Important Role in Determining the Relative Stabilities of the Open and Closed States**

The shifts in the voltage-dependence curve of channel activation produced by mutations of the S4 hydrophobic residues may be explained in terms of their effects on the stability of the open and closed states of the channel. The fraction of channels in the open state ($G/G_{max}$) at membrane potential V is given by the equation:

$$G / G_{max} = [ 1 + \exp (( \Delta G - zeV ) / kT )]^{-1}$$

where $\Delta G$ is the difference between the free energy of the open and the closed state, z is the gating charge valence, e is the elementary charge, k is Boltzmann's constant, and T is the absolute temperature (Hille, 1984). Mutations that alter the difference between the free energy levels of the open and closed states ($\Delta G$) will cause the transition between these two states to take place at a different range of membrane potentials and thereby shift the voltage-dependence curves along the voltage axis. This explanation also applies to situations involving more than two states, as in the case of

voltage-dependent activation of the *Shaker* potassium channel (Zagotta et al., 1989b; Zagotta and Aldrich, 1990a).

The hydrophobic substitution mutations are expected to affect only short-range hydrophobic interactions of the S4 sequence and its immediate surrounding. In all of the mutations tested, the side chains involved are composed of methyl groups, which should minimize any potential long-range electrostatic interactions involving induced dipoles. In most of our mutants, a leucine residue is replaced with alanine which should take up the space of the $\beta$ carbon of the leucine side chain (Ponder and Richards, 1987), so that the perturbation of structure should be minimal. Whereas mutations of a key cysteine residue of the acetylcholine receptor altered the gating in such a way that the alteration of the energy barrier for a single closing rate constant is proportional to the size of the substituted side chain (Lo et al., 1991), structural analysis of a mutant T4 lysozyme has revealed that even though the replacement of leucine 133 with phenylalanine (with a large side chain) was designed to fill one of the largest cavities that exist in the folded structure of the native protein, it caused displacement of neighboring side chains and forced the introduced amino acid side chain to adopt non-optimal dihedral angles, thereby compromising the thermal stabilities of the protein (Karpusas et al., 1989). Thus, it may be generally less intrusive to replace a

residue with one with a smaller side chain. In fact, the hydrophobic substitution mutations of the S4 have been found to affect the voltage-dependent but not the voltage-independent channel properties, indicating that they did not grossly alter the channel protein structure. Therefore, the large shifts caused by these mutations indicate that the interactions between the S4 sequence and its immediate surrounding contribute significantly to the relative stabilities of the open and closed states.

**The S4 Sequence is Likely to be Buried Within the Channel Protein**

How might our findings be explained in light of what is known of protein structure? If the S4 sequence indeed spans the membrane, it would most likely be surrounded by other membrane-spanning segments of the channel, so that the basic residues of the S4 could form salt bridges with acidic residues in these other membrane-spanning segments. Given the observed close packing within globular proteins (Richards, 1977; Ponder and Richards, 1987) and within the bacterial photosynthetic reaction center (Yeates et al., 1987; Komiya et al., 1988), it would not be very surprising if the hydrophobic residues of the S4 sequence were in contact with hydrophobic residues in the membrane spanning segments that surround the

S4 sequence. The proposal that channel activation is triggered by movement of the S4 would then imply that the hydrophobic and electrostatic interactions between the S4 sequence and other parts of the channel are not identical in the closed and the open states. Indeed, each time we mutated a hydrophobic residue in the S4 sequence there was a dramatic shift of the voltage-dependence curves. Similar conservative substitutions of hydrophobic residues in the protein interior are often found to be without strong effects (Lesk and Chothia, 1980; Bowie et al., 1990). Moreover, mutations of highly conserved leucines in proposed membrane spanning segments other than the S4 did not have strong effects on the voltage-dependence of gating. Thus the S4 sequence is intimately involved in voltage-dependent activation, probably because it is buried within the channel protein and its movement during channel activation changes its interactions with the rest of the protein. If the movement of the S4 sequence corresponds to the observed translocation of about 8 electronic charges across the membrane (Zagotta and Aldrich, 1990a), it would be much greater than the moderate relative movements that have been observed within a protein subunit (Perutz, 1979; Royer et al., 1990). One possibility to account for the proposed large displacement of the S4, is that part of the S4 structure is at the interface between subunits, so that the proposed movement of the S4 sequence not only involves movement of the

S4 relative to other parts of the same subunit, but also relative movements of adjacent subunits. The extent of tilting and sliding seen between subunits of the gap junction or the acetylcholine receptor (Unwin, 1989) could account for a major part of the postulated outward movement of the S4 basic residues.

**Other Mutations that Shift the Voltage-Dependence Curves**

It has been noted that several of the recently cloned potassium channels contain a "leucine-zipper" motif, a tandem repeat of five leucines at every seventh position beginning partway through the S4 sequence (McCormack et al., 1989). To test their proposal that this "leucine-zipper" is involved in subunit assembly, McCormack et al. (1991a) examined mutations of these five leucine residues in the *Shaker* potassium channel and found that the mutations did not affect the assembly of subunits. Instead, they found that the first two leucines (the last two leucines in the S4), when mutated to valine, produce much larger shifts in the voltage-dependence curve of channel activation than mutations of the last three leucines. Their conclusion that:

*"the interactions mediated by the heptad leucines strongly influence the transduction of charge movement into channel opening and closing",*

however, seems unjustified, since mutations of each of the leucine residues in the *Shaker* S4 sequence (Table 1) as well as a leucine in the second S4 sequence of the rat brain sodium channel (Auld et al., 1990) shifted the voltage-dependence curves, regardless of their positions relative to the "leucine-zipper".

It is important to note that mutations that shift the voltage-dependence curves are not necessarily restricted to mutations of the voltage sensor. For example, mutations that alter the surface potential or local electrical field within the channel molecule are expected to shift the voltage-dependence curves (MacKinnon et al., 1989). Since extracellular calcium stabilizes the closed state of the *Shaker* potassium channel and potassium channels in the squid (Armstrong and Miller, 1990; Armstrong and Lopez-Barneo, 1987), and other divalent cations cause a shift of the voltage-dependence curve (Mayer and Sugiyama, 1988), mutations which affect interactions between divalent cations and the channel molecule could also alter the relative stabilities of the open and closed states. Moreover, if the conformational changes that take place during the transition between the

closed and open states involve movements of the voltage sensor and perhaps subsequent movements of other parts of the channel, it is conceivable that mutations of residues in several parts of the channel can alter the relative stabilities of the open and closed states. Mutations in different parts of the channel have in fact been found to cause moderate shifts in the voltage-dependence curve of activation (Salkoff and Wyman, 1981; Gautman and Tanouye, 1990; Lichtinghagen et al., 1990; VanDongen et al., 1990; Zagotta and Aldrich, 1990b). However, our studies indicate that the S4 sequence appears to be more critically involved in voltage-dependent activation; whereas a sample of five hydrophobic substitution mutations in other proposed membrane spanning segments revealed mild or no effects on the voltage-dependence, each of the six hydrophobic substitution mutations in the S4 sequence had much stronger effects.

In summary, we have shown by mutagenesis that the S4 sequence of the *Shaker* potassium channel is centrally involved in the process of voltage-dependent activation. Previous mutagenesis studies of basic residues in this S4 sequence, designed to test the prediction of the S4 model in which the S4 basic residues function as the voltage sensor, revealed strong and specific effects of these mutations on the voltage-dependence of activation (Papazian et al., 1991). These effects, however, could not be explained by

simply considering the electrostatic interactions between the S4 basic residues and the membrane electric field. By making conservative mutations of hydrophobic residues, we now show that the hydrophobic interactions between the S4 sequence and its immediate surrounding play a significant role in voltage-dependent activation of the channel. Whether the S4 sequence spans the membrane as proposed in the S4 model (Greenblatt et al., 1985; Kosower, 1985; Catterall, 1986; Guy and Seetharamulu, 1986; Noda et al., 1986) has to be determined by biochemical studies. Nonetheless, our observations indicate that the S4 sequence is likely to be buried within the channel molecule, so that most of its hydrophobic residues interact closely with other parts of the channel protein. These interactions appear to be very important in determining the relative stabilities of the open and closed states of the channel.

## FIGURES AND TABLES

**FIGURE 1**

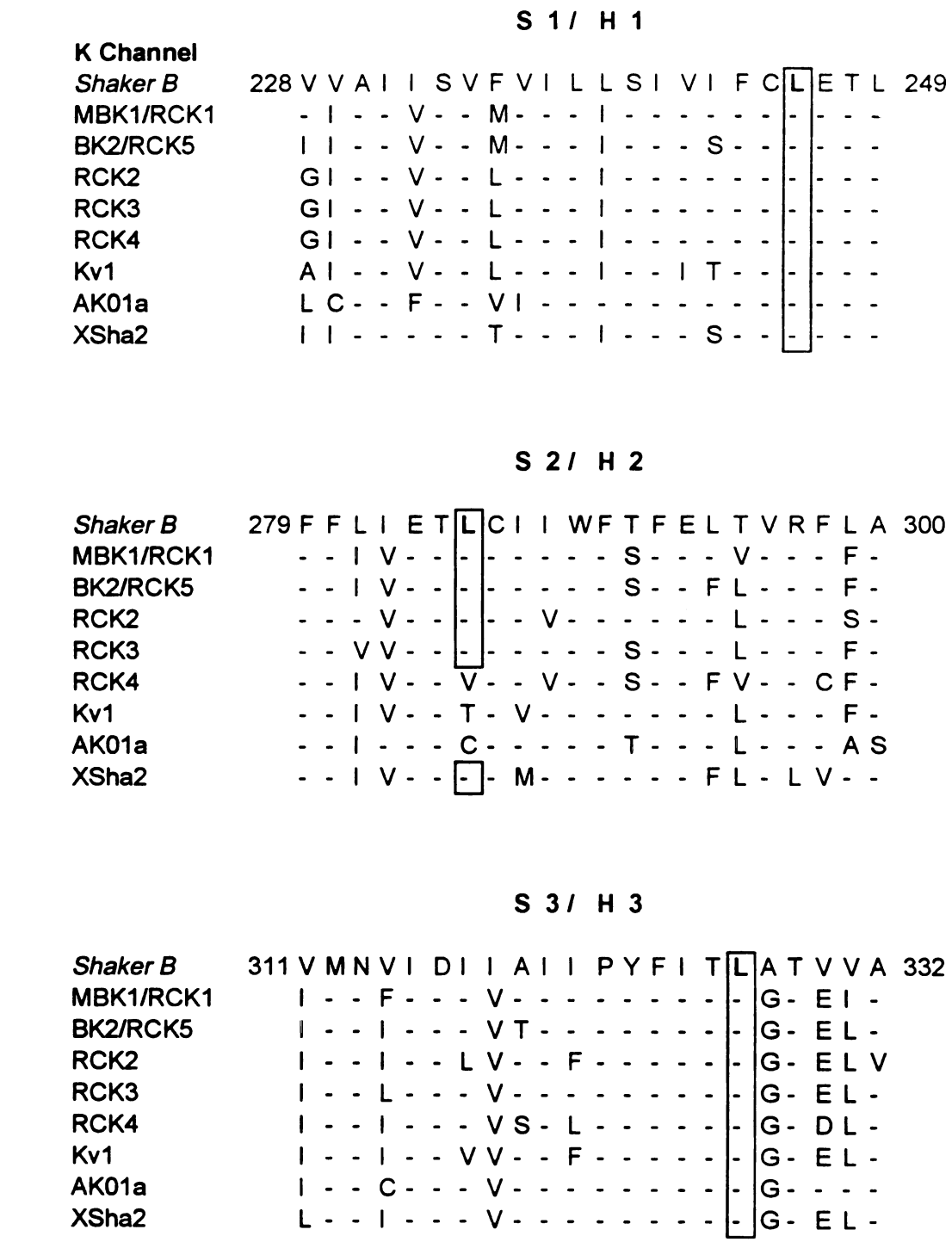


**FIGURE 1 Continued**

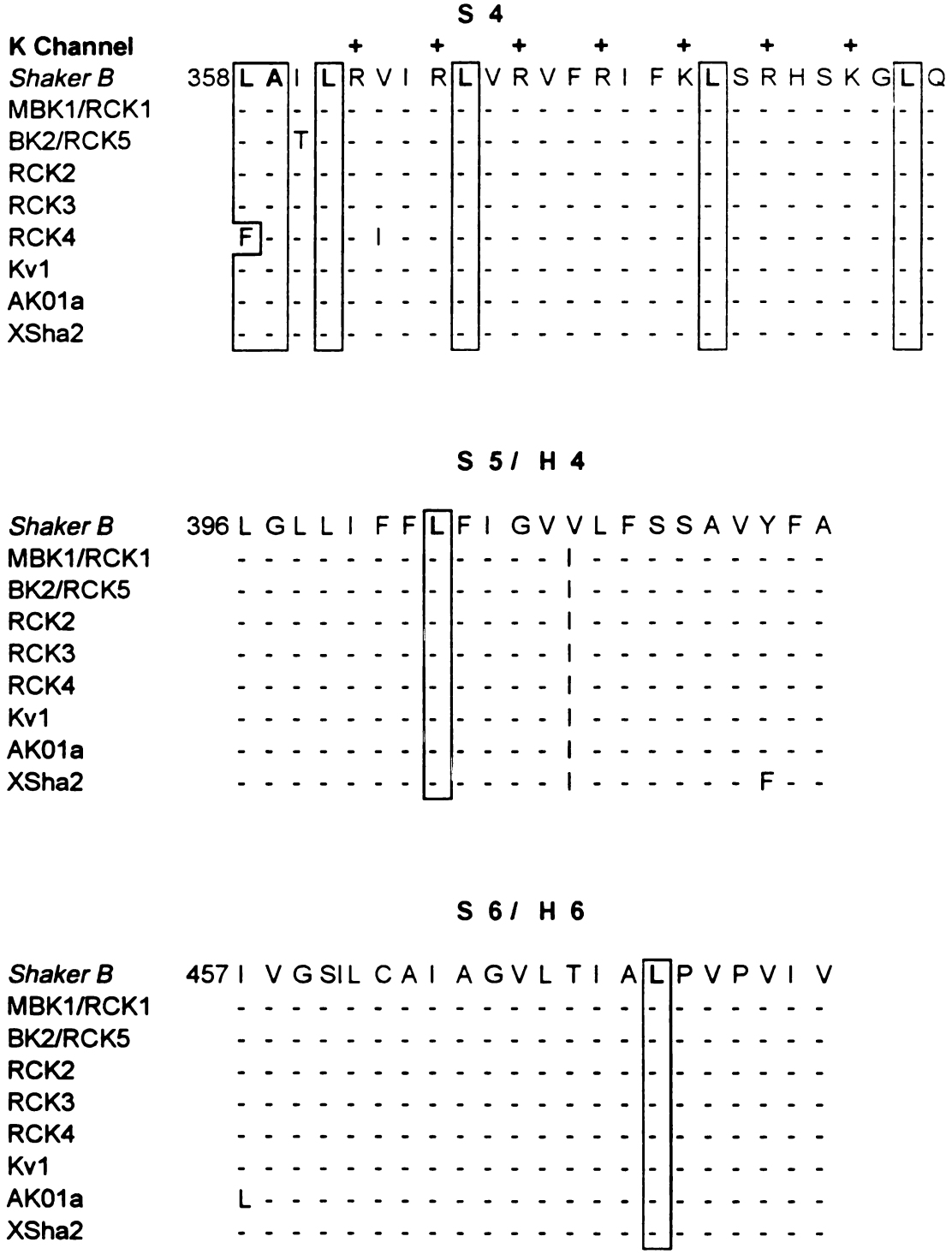


**FIGURE 1 Hydrophobic Residues in the S4 Sequence and Other Proposed Membrane Spanning Segments that are Highly Conserved Among the *Shaker* Subfamily of Potassium Channels Were Selected for Site-Directed Mutagenesis.**

The mutated residues are boxed in the sequence alignment. The first and last amino acid residue are numbered in each proposed transmembrane segment on the *Shaker* B clone. The S4 sequence shown here starts at amino acid 358 in the *Shaker* B clone and ends at position 383. The '+' represents the basic residues. References for the potassium channels are: *Shaker* B, Schwarz et al., 1988; MBK1, Tempel et al., 1988; RCK2, Grupe et al., 1990; BK2, McKinnon, 1989; RCK1,3-5, Stühmer et al., 1989b; Kv1, Swanson et al., 1990; XSha2, Ribera, 1990; AK0la, Pfaffinger et al., 1991.

Hydrophobic Mutations in S4 Alter Voltage Dependence

FIGURE 2

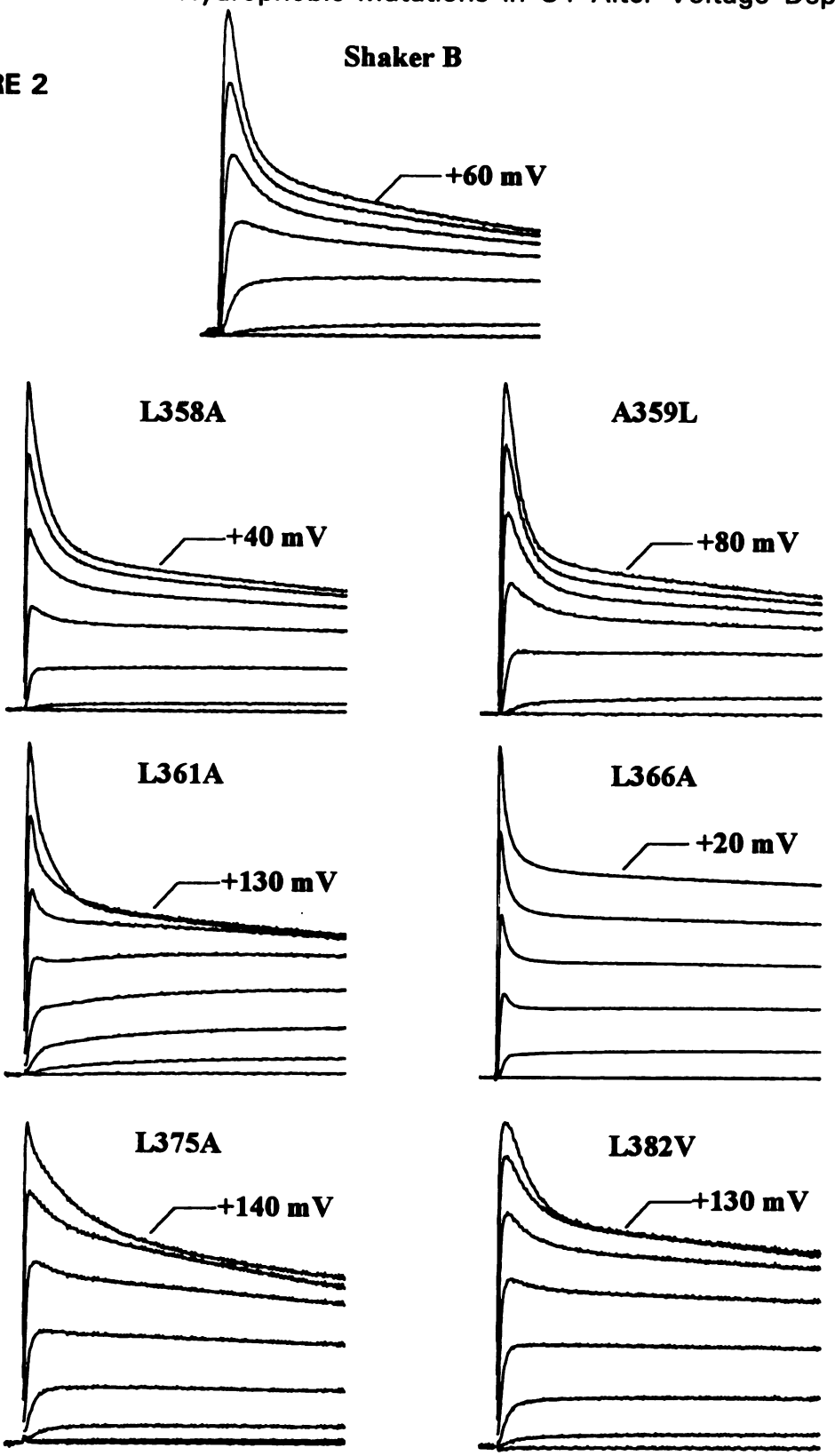


**FIGURE 2 Currents Through Wild-type *Shaker* B Channels and S4 Hydrophobic Substitution Mutants.**

Families of normalized currents elicited by 500 msec test pulses in 20 mV increments, delivered every 30 seconds, to different voltages ranging from -100 mV to +140 mV depending on the mutant. Peak current was 2.5$\mu$A at +60 mV for *Shaker* B; 3.5 $\mu$A at +40 mV for L358A; 8.4 $\mu$A at +80 mV for A359L; 5.0 $\mu$A at +130 mV for L361A; 7.2 $\mu$A at +20 mV for L366A; 3.5 $\mu$A at +140 mV for L375A; 3.4 $\mu$A at +130 mV for L382V. Holding potential was -100 mV. The recording temperature was 11 ± 1°C. The larger amplitude of the second component of inactivation in the mutants L361A, L375A and L382V may be partly due to contaminating potassium currents endogenous to the oocyte at the larger depolarizations required to activate these mutant channels.

**FIGURE 3**

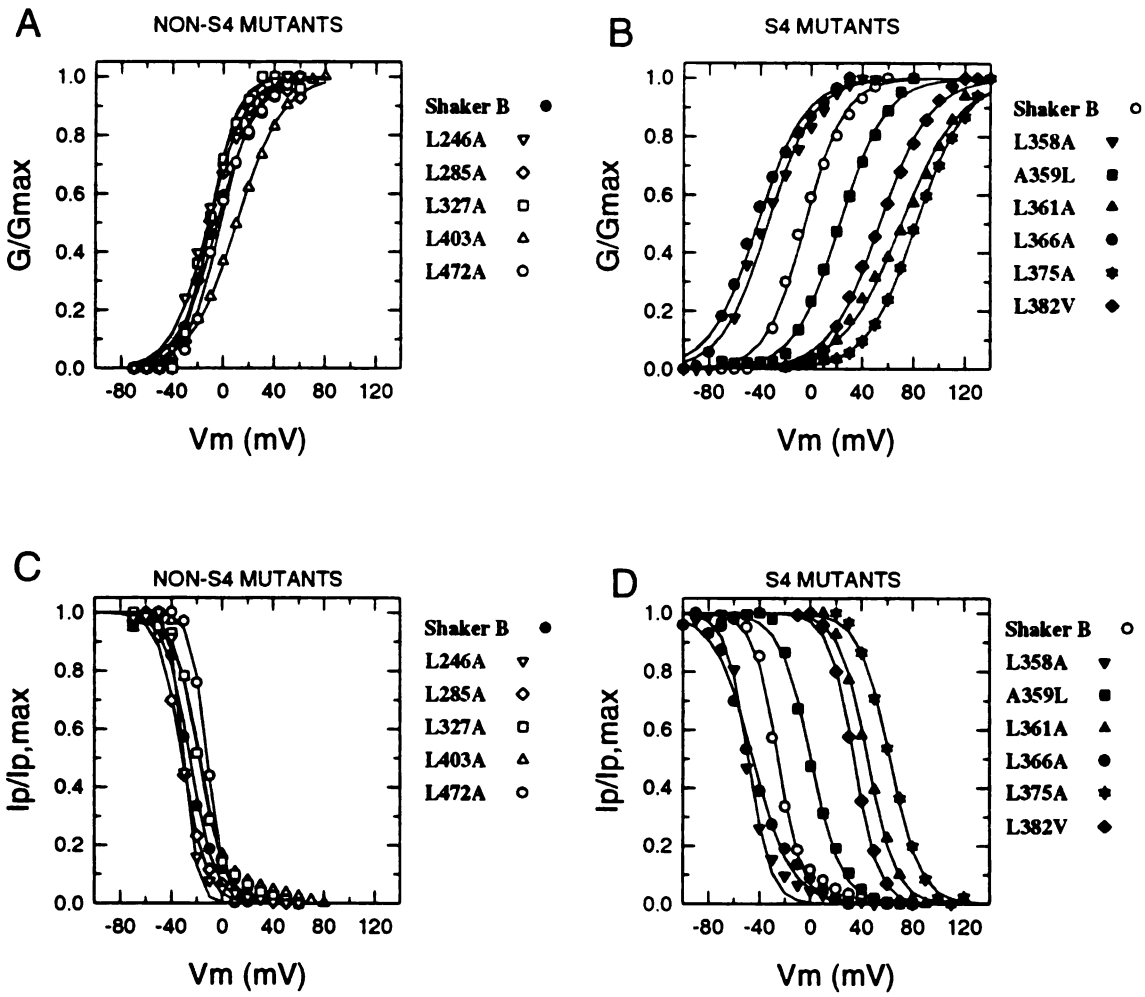


**FIGURE 3 Voltage-Dependence of Activation and Macroscopic Inactivation of Currents Through S4 Mutant and non-S4 Mutant Channels.**

**(A)** Normalized conductance-voltage curves for *Shaker* B and the non-S4 mutants. **(B)** Normalized conductance-voltage curves for *Shaker* B and the S4 mutants. **(C)** Normalized prepulse inactivation curves for *Shaker* B and the non-S4 mutants. **(D)** Normalized prepulse inactivation curves for *Shaker* B and the S4 mutants. To determine the conductance, the peak amplitude of the leak-subtracted current was divided by the driving force across the membrane. The conductance values were normalized with respect to the maximal conductance and plotted versus the membrane potential. The symbols represent data from single representative experiments. The solid curves are theoretical (see Methods). For the prepulse inactivation curves, a 500 msec prepulse at the indicated membrane potential was followed by a 100 msec test pulse to +40 mV for non-shifted mutants or to potentials between +20 mV and +120 mV depending on the extent of the shift in the S4 mutant channels. The peak current during the test pulse was normalized to the maximal peak current with no prepulse. The solid curves are theoretical fits to the Boltzmann equation (see Methods).

**FIGURE 4**

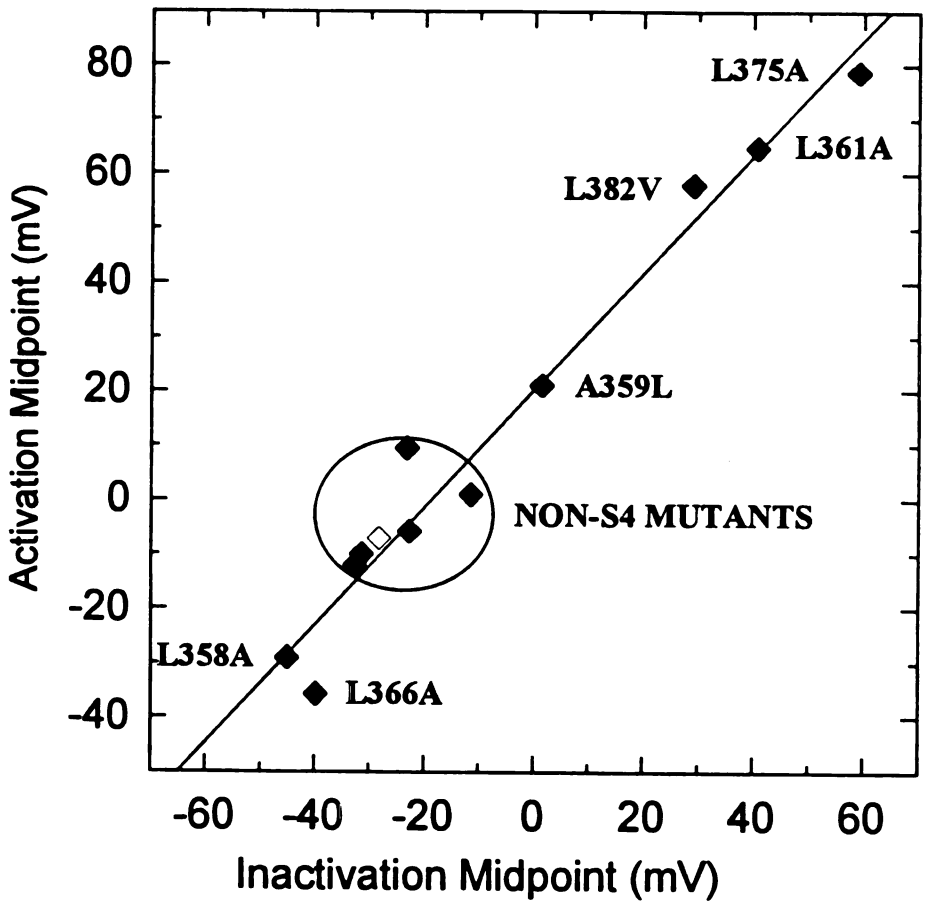


**FIGURE 4 Channel Activation and Inactivation are Coupled.**

The mean of the midpoints of the conductance-voltage curves (activation) is plotted against the mean of the midpoints of the prepulse inactivation curves (see Table 1 for the means). The circled points are the non-S4 mutants along with *Shaker* B (open symbol). The straight line was fit to the data points by linear regression with a slope of 1.07 and a correlation coefficient of 0.98.

**TABLE 1 Steady-State Activation and Inactivation Parameters**

| | ACTIVATION | | INACTIVATION | | |
|---|---|---|---|---|---|
| | $V_{0.5}$(mV) | b(mV) | $V_{0.5}$(mV) | b(mV) | N |
| *Shaker B* | -7.1 ± 3.7 | 15.8 ± 2.6 | -28.3 ± 4.4 | 7.2 ± 2.0 | 6 |
| L246A | -12.0 ± 3.4 | 14.1 ± 1.9 | -32.7 ± 3.1 | 6.8 ± 1.9 | 4 |
| L285A | -10.1 ± 4.4 | 15.4 ± 3.3 | -31.4 ± 3.1 | 8.3 ± 1.1 | 6 |
| L327A | -5.9 ± 5.1 | 12.8 ± 2.1 | -22.7 ± 5.1 | 7.8 ± 1.8 | 4 |
| L358A | -29.0 ± 3.4 | 19.5 ± 1.5 | -45.4 ± 3.7 | 9.5 ± 0.8 | 4 |
| A359L | 21.0 ± 1.5 | 17.9 ± 1.6 | 1.6 ± 3.0 | 11.1 ± 1.8 | 4 |
| L361A | 64.8 ± 7.3 | 22.7 ± 1.1 | 40.8 ± 4.1 | 8.5 ± 0.6 | 4 |
| L366A | -35.8 ± 3.6 | 18.8 ± 0.8 | -39.8 ± 3.5 | 15.7 ± 0.5 | 5 |
| L375A | 78.7 ± 4.7 | 18.1 ± 1.0 | 59.2 ± 5.9 | 10.4 ± 1.2 | 7 |
| L382V | 58.0 ± 3.7 | 20.8 ± 1.3 | 29.2 ± 3.2 | 9.0 ± 0.8 | 3 |
| L403A | 9.5 ± 3.6 | 18.6 ± 1.8 | -23.2 ± 3.6 | 9.4 ± 0.5 | 6 |
| L472A | 0.9 ± 2.9 | 13.5 ± 1.4 | -11.4 ± 0.7 | 6.8 ± 0.5 | 4 |

**TABLE 1 Steady-State Activation and Inactivation Parameters**

Normalized conductance-voltage and prepulse inactivation curves were fitted with the Boltzmann equation to obtain values for the midpoint potential $V_{0.5}$ and the slope parameter k (see Methods). For each parameter, the mean, standard deviation, and number of oocytes (N) are given. Much larger shifts of the voltage-dependence curves are produced by the S4 mutations than the non-S4 mutations.

**TABLE 2 Fast Macroscopic Inactivation Kinetics and Reversal Potentials**

| | $\tau$ | $V_m$ | N | $V_r$ | $V_r$ | N |
|---|---|---|---|---|---|---|
| | | | | 20 mM $K^+$ | 40 mM $K^+$ | |
| | (msec) | (mV) | | (mV) | (mV) | |
| *Shaker B* | 17.7 ± 1.1 | 60 | 4 | -35 ± 2 | -21 ± 2 | 4 |
| L358A | 20.0 ± 2.7 | 50 | 8 | -36 ± 3 | -21 ± 5 | 5 |
| A359L | 22.0 ± 3.4 | 80 | 4 | -37 ± 3 | -22 ± 4 | 4 |
| L361A | 25.1 ± 5.6 | 100 | 4 | -35 ± 3 | -21 ± 1 | 4 |
| L366A | 11.8 ± 1.1 | 10 | 4 | -36 ± 2 | -20 ± 2 | 5 |
| L375A | 22.9 ± 2.9 | 140 | 3 | ND | ND | - |
| L382V | 30.3 ± 2.7 | 100 | 6 | -34 ± 1 | -20 ± 1 | 3 |

**TABLE 2 Fast Macroscopic Inactivation Kinetics and Reversal Potentials of the S4 Mutants**

Currents evoked by 500 msec depolarizing pulses (from a holding potential of -100 mV to the membrane potential $V_m$, which is at or near Gmax as measured from Figure 3) were used to measure the time constant of the fast-inactivating component. Exponential fits were done using the least-squares minimization program of pCLAMP. The reversal potential of the current, $V_r$ (mV), was determined in 20 mM and 40 mM external potassium from measurements of tail currents evoked by steps in 5 mV increments in the region of reversal potential. The change of extracellular potassium concentration was accompanied with changes of the extracellular sodium concentration so as to maintain the extracellular concentration of monovalent cations constant. $V_r$ could not be determined for L375A since neither inward nor outward tail currents were observed in 20, 40, or 89 mM external potassium in this mutant, which did not begin to activate until about +40 mV. These voltage-independent properties are fairly similar between currents of the S4 mutants and the wild-type *Shaker* B. The mean, standard deviation, and number of oocytes (N) are given. Recordings were done at 11 ± 1°C. (ND: not determined).

# CHAPTER III

## Evidence That the S6 Segment of the *Shaker* Voltage-Gated Potassium Channel Comprises Part of the Pore

## ABSTRACT

Potassium channels are highly selective and allow the rapid flux of potassium ions through their pore. Several studies have implicated the H5 (P region or SS1-SS2) segment as part of the pore in voltage-gated ion channels. The proposal that H5 spans at least 80% of the electric potential drop across the potassium channel pore is based on altered internal tetraethylammonium sensitivity from mutations of H5 residues that are 100% conserved among potassium channels having diverse tetraethylammonium sensitivities. We report that the S6 segment is also involved in potassium ion permeation and in governing the sensitivity to internal tetraethylammonium and barium. Transplanting the S6 segment of NGK2 into *Shaker* caused this S6 chimera to adopt the single-channel conductance and sensitivity to internal tetraethylammonium and barium ions from the NGK2 channel. The differences between NGK2 and *Shaker* in external tetraethylammonium sensitivity, but not single-channel conductance, could be attributed to their differences in the H5 sequence. Three nonconserved S6 residues have been found to affect either single-channel conductance or internal tetraethylammonium sensitivity.

## INTRODUCTION

Previous studies have indicated that the H5 (also known as SS1-SS2 or P region; Guy and Conti, 1990; Hartmann et al., 1991) segment forms the pore (Yool and Schwarz, 1991; Hartmann et al., 1991; Yellen et al, 1991). Nevertheless, there are indications that H5 does not comprise the entire pore-lining structure. First, although the sensitivity to internally applied TEA is altered by mutations of a particular residue, threonine 441, in the H5 region (Yellen et al., 1991), this threonine residue is 100% conserved in all cloned voltage-gated potassium channels even though they show a wide range of sensitivities to internal TEA (Figure 5, Chapter I) (Yellen et al., 1991; Hartmann et al., 1991; Kirsch et al., 1992; Taglialatela et al, 1991; Kirsch et al., 1991). Therefore, non-conserved amino acids outside of the H5 segment are probably responsible for the different sensitivities to internal TEA. Second, the potassium channel is postulated to contain a long, narrow pore with multiple potassium binding sites (Hille, 1992), but the H5 segment as an anti-parallel beta sheet structure (Yellen et al., 1991), may be too short to contain all of the postulated potassium binding sites. To identify

additional pore-lining structures, we examined the amino acid sequence of the proposed transmembrane segments for conserved domains among all of the cloned voltage-gated potassium channels. Of the proposed transmembrane segments, the sixth segment (S6) is the most highly conserved (Figure 6, Chapter I).

To determine whether the S6 segment forms part of the permeation pathway, we constructed chimeric potassium channels between *Shaker B* (Schwarz et al., 1988) and NGK2 (Yokoyama wt al., 1989), and measured several pore properties that differ between these two potassium channels which are from different subfamilies (Hille, 1992). The NGK2 channel has a single-channel conductance larger than that of *Shaker B* (Yellen et al., 1991; Hartmann et al., 1991). In addition, the NGK2 channel is approximately two orders of magnitude more sensitive to external block by tetraethylammonium (TEA), and over an order of magnitude less sensitive to internal block by TEA than *Shaker B* (Yellen et al., 1991; Hartmann et al., 1991). The S6 chimera was constructed by transplanting an S6-containing segment of NGK2 to replace that of *Shaker B* (Figure 1). The construct also contains a deletion of amino acids 6 through 46 (ShBΔ) in the amino terminus (Hoshi et al., 1990). This amino terminal deletion removes fast inactivation which

would otherwise interfere with the action of open channel blockers such as TEA and barium ions, and does not affect potassium permeation (Hoshi et al., 1990). If the S6 segment forms part of the permeation pathway, then we would expect several of the pore properties of the S6 chimera to resemble those of NGK2 rather than ShBΔ.

We have determined that the S6 segment of the *Shaker* voltage-gated potassium channel is involved in the blockade of the pore by cytoplasmically applied TEA and barium ions, as well as the permeation rate of potassium ions. Thus the S6 segment appears to contribute to an internal and deep portion of the pore structure. In contrast to the view that the H5 segment forms the entire pore and fully determines the conduction properties of potassium channels (Mackinnon, 1991a; Miller, 1991; Miller, 1992a; Miller, 1992b), our results suggest that H5 is not the only pore forming region of the channel. Rather, the long pore of voltage-gated potassium channels is likely to be formed by multiple structural components including the H5 sequence, the S4-S5 intracellular loop (Isacoff et al., 1991; Slesinger et al., 1993), and the S6-containing segment.

## METHODS

### Site Directed Mutagenesis

Restriction sites that flank the S6 segment but do not alter the amino acid sequence were introduced into ShBΔ. The two restriction sites *Nru I* and *Hind III* were introduced by oligonucleotide directed mutagenesis. Oligonucleotide site-directed mutagenesis was performed using single stranded cDNA template in Bluescript $SK^-$ as described by Sayers and Eckstein (1989). The wild-type strand was removed with Exonuclease III after nicking with *NciI*, producing an efficiency of mutagenesis greater than 90%. Mutagenized DNA was characterized by DNA sequencing. In order to help insure that no second site mutations were created in the procedure, the following controls were done: (1) all of the mutant clones were sequenced in a region of 100 to 200 bases on either side of the mutation, (2) at least two independent clones were isolated for each mutant and were found to have the same electrophysiological phenotype. The mutated DNA was then cut with the two restriction enzymes *Hind III* and *Nru I* and gel purified with agarose gel electrophoresis. For the cassette mutagenesis, a double stranded

DNA segment was constructed with single stranded complementary synthetic oligonucleotides containing the coding sequence of the corresponding region from the NGK2 clone, ligated into the ShBΔ backbone and verified by sequencing. The single and multiple-site mutants were also made with synthetic oligonucleotides and similarly sequenced. The mutant clones were sequenced using either single stranded or double stranded DNA sequencing (Sanger et al., 1977; Mierendorf and Pfeffer, 1987). The H5 chimera was similarly constructed by cassette mutagenesis using the restriction sites *Nsi I* and *Hind III* which flank the H5 region of ShBΔ (amino acid 433 to 460). The corresponding H5 sequence of NGK2 (Yokoyama et al., 1989), identical to that transplanted into DRK1 in a previous study (Hartmann et al., 1991), was used to replace the H5 of ShBΔ.

### *In Vitro* Transcription

Once the mutant clones were identified by sequencing, the template DNA (approx. 2 μg) was linearized with the restriction enzyme *EcoO109I*. RNA was transcribed using 0.5 mM each of rATP, rCTP, rGTP, rUTP and the cap analog diguanosine triphosphate (Pharmacia) in a standard transcription buffer containing 40 mM Tris (pH 8.0), 8 mM $MgCl_2$, 2 mM spermidine, 50 mM NaCl and 75 mM dithiothreitol. 50 units of T3 RNA polymerase was

used in the presence of 80 units of RNase inhibitor (Promega) for 2 hours at 37°C. The template DNA was then digested using 2 units of RNase-free DNase (Promega) for 30 minutes at 37°C. The RNA was purified using phenol/chloroform extractions followed by ethanol precipitation. To confirm the size and integrity of the RNA produced, denaturing gel electrophoresis was performed using one tenth of the transcription reaction and the migration compared to an RNA size ladder (BRL). The RNA was denatured in a solution of 50% formamide and 2.2 M formaldehyde and heated at 65°C for 2 minutes prior to loading onto a 0.7% agarose, 6.7% formaldehyde gel. The RNA was stored at -70°C in sterile water.

**Oocyte Injection**

Adult female *Xenopus laevis* were anesthetized by immersion in ice water for 1 hour followed by surgical removal of several ovarian lobes. Oocytes were manually separated from the ovarian connective tissue prior to injection. Stage V and VI oocytes (Dumont, 1972) were injected with approximately 50 nl of RNA (1-2 mg/ml) and incubated in modified Barth's saline (MBS) containing 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 10 mM HEPES (pH 7.5), 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, and 0.41 mM $CaCl_2$ (Gurdon and Wickens, 1983) at 18°C. Twenty four hours following

injection, oocytes were incubated in MBS with 2 mg/ml collagenase (cls 3, Worthington) for 2 hours with gentle shaking. This treatment helps remove the overlying connective tissues and follicular cells. Oocytes were incubated in MBS supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μg/ml gentamicin for 2-4 days before recording.

**Electrophysiological Recordings**

To simplify the kinetic analysis and measurements, we used an amino-terminal deletion mutant of *Shaker B*, ShBΔ, which does not exhibit fast inactivation (Hoshi et al., 1990). For whole cell experiments, ionic currents were recorded using a standard two-microelectrode voltage clamp circuit. Data acquisition and analysis were performed on a 80386 based micro-computer using the program pCLAMP and an A/D converter (Axon Instruments). Voltage and current micro-electrodes were filled with 3M KCl and had resistances of less than 2MΩ. Series resistance, approximated at 500 ohms (Papazian et al., 1991), was compensated for. Current signals were samples at 5 kHz and low-pass filtered at 1 kHz with an 8-pole Bessel filter. Leakage currents were subtracted by adding responses to scaled 20 mV hyperpolarizing pulses (P/4 method, Bezanilla and Armstrong, 1977). The *Shaker* potassium channel has an approximately linear open channel

current-voltage relation between +30 and +80 mV (MacKinnon and Yellen, 1990) and between +20 and +120 mV (E. Isacoff, personal communication). Experiments for the external TEA sensitivity were performed on RNA injected, voltage clamped *Xenopus* oocytes using a two-electrode voltage clamp, at a holding potential of -100 mV. The external solutions contained: TEACl of various concentrations as indicated, NaCl of varying concentrations so as to maintain the total concentration of NaCl and TEACl at 88 mM, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, and 10 mM HEPES (pH 7.5, NaOH). The curves were fit to the equation:

$$\text{Fractional Current} = 1 / ( 1 + [TEA] / K_i).$$

For the internal TEA or barium sensitivity, experiments were done on excised inside-out patches from a holding potential of -100 mV. TEACl or barium chloride was applied by perfusing the intracellular side of the patch with a perfusion pipette brought into close proximity to the membrane. For single-channel recordings, single-channel currents were recorded from excised inside-out patches using a List EPC-7 amplifier, sampled at 5 kHz and filtered at 1 kHz. The external (pipette) solution contained 98 mM KCl, 1 mM $MgCl_2$, 0.3 mM $CaCl_2$, 10 mM HEPES (pH 7.1, KOH). The internal (bath) solution contained 98 mM KCl, 0.5 mM $MgCl_2$, 1 mM EGTA, and 10 mM HEPES (pH

7.1, KOH) (20-23°C). Holding potential was -100 mV and the membrane was depolarized to +100 mV for 320 msec. Single-channel current amplitudes were measured from amplitude histograms and were leak subtracted using blank traces which contained no channel openings.

To control for the possibility that the chimeric mutation affected protein structure and altered channel function in a non-specific manner, we measured from the S6 chimera and the ShBΔ channels, two separate properties of channel gating: (1) the voltage-dependence of activation and (2) the maximum open channel probability. The voltage-dependence of activation was measured from the size of macroscopic tail currents following activation voltage pulses to various voltages. The voltage-dependence of the normalized tail current was fitted to a Boltzmann distribution to determine the $V_{mid}$ (mV) and slope (b) (mV/e-fold change). The theoretical curves fitting the conductance-voltage relations were determined using an iterative non-linear regression fitting procedure to the Boltzmann equation:

$$G / G_{max} = 1 / [1 + \exp((V_{mid} - V_m) / b)]$$

where $V_m$ is the membrane potential. From this fit, a slope parameter (b) and the midpoint potential ($V_{0.5}$) are obtained. Values for ShBΔ: $V_{mid}$ = -45 ± 2 mV, slope = 3.7 ± 0.7 mV/e-fold, N = 8; Values for the S6 chimeric

channel: $V_{mid}$ = -44 ± 2 mV, slope = 3.9 ± 0.4 mV/e-fold, N = 3 (mean ± SD). The tail currents at -100 mV were fitted with a single exponential using the program Clampfit (Axon Instruments). The time constants are not significantly different for ShBΔ: 3.4 ± 0.5 msec., N = 5, and the S6 chimera: 3.2 ± 0.7 msec., N = 4, mean ± SD. The open channel probability was measured at +100 mV with each digitized point counted as either open or closed according to a 50% current threshold. $P_{open}$ for ShBΔ: 0.88 ± .03, N = 4; $P_{open}$ for the S6 chimeric channel: 0.92 ± 0.02, N = 4 (mean ± SD). All experiments were carried out at room temperature, 21 ± 1°C.

## RESULTS

### Transplanting Single-Channel Conductance from NGK2 to *Shaker* with the S6 Segment

Transplanting the S6 region (Figure 1) from NGK2 into ShBΔ increased the single-channel conductance by four-fold (Figure 1, Table 1), from 11 pS to 44 pS. This increase in single-channel conductance was unexpected, given that the difference in the single-channel conductance between NGK2 (27 pS) and DRK1 (8 pS) had been attributed to the H5 sequence in a similar analysis of chimeric potassium channels (Hartmann et al., 1991). The identical H5 segment of NGK2, which confers a large conductance when transplanted into DRK (Hartmann et al., 1991), did not alter the single-channel conductance when transplanted into ShBΔ, even though this H5 chimera exhibited an external TEA sensitivity ($K_i$ = 0.6 mM) similar to that of NGK2 ($K_i$ = 0.3 mM) rather than that of ShBΔ ($K_i$ = 21 mM) or the S6 chimera ($K_i$ = 23 mM) (Figure 2a, Figure 3; Table 1). Thus, both the H5 and S6 segment appear to limit the conductance, perhaps by

forming a tight constriction, but only the H5 segment contains the external binding site for TEA. The small conductance of DRK1 is probably due to its H5 segment restricting potassium flux, whereas the small conductance of ShBΔ appears to arise primarily from a limiting S6 segment.

**Alteration of TEA and Barium Blockade by the S6 Mutations Identifies the Inner Mouth and Vestibule of the *Shaker* Channel**

The S6 chimera not only increased the single-channel conductance but it also altered the sensitivity to internally applied TEA and barium ions. While TEA blocks ion conduction by occluding the mouth of the pore (Armstrong and Binstock, 1965; Armstrong, 1966; Armstrong, 1969; Armstrong, 1971; Armstrong and Hille, 1972), barium ions block the potassium channel pore by interacting with potassium binding sites at various depths in the pore (Armstrong and Taylor, 1980; Eaton and Brodwich, 1980; Armstrong et al., 1982; Vergara and Latorre, 1983; Benham et al., 1985; Miller et al., 1987; Neyton and Miller, 1988a; Neyton and Miller, 1988b; Hille, 1992; Taglialatela et al., 1993), probably due to the similar crystal radii of barium and potassium ions (1.35 Å vs. 1.33 Å,

respectively (Hille, 1992)). The presence of 50 $\mu$M internal barium ions produced a time- and voltage-dependent block of the NGK2 potassium channels (Figure 2 g, h) due to the relatively slow rate of the barium block of the open channel (Armstrong and Taylor, 1980; Eaton and Brodwich, 1980; Armstrong et al., 1982; Vergara and Latorre, 1983; Miller et al., 1987; Neyton and Miller, 1988a; Neyton and Miller, 1988b;). Compared to the block of NGK2 channels, the barium block of ShBΔ channels was much slower and less effective (Figure 2 c, d). The barium block of the S6 chimera was more similar to that of NGK2 than ShBΔ (Figure 2 e, f). In particular, the barium block showed much greater voltage-dependence for NGK2 and the chimeric channel than for ShBΔ (Figure 2 c-i). Thus, barium appears to penetrate further into the pore of the NGK2 and chimeric channels, so that its binding to the channel pore is influenced by the electric potential across the membrane. This suggests that transplanting the S6-containing segment of NGK2 into ShBΔ has either introduced a barium binding site in the pore or alternatively has exposed such a site by reducing an energy barrier to barium entry. In addition, the internal TEA sensitivity of the S6 chimera was similar to that of NGK2 rather than that of ShBΔ (Figure 2 b; Table 1), suggesting that the internal mouth or vestibule of the pore of the S6 chimera resembles that of NGK2.

**Alteration of Pore Properties Without Affecting Voltage-Dependent or Kinetic Properties of Channel Gating**

To control for the possibility that the chimeric mutation affected protein structure and altered channel function in a non-specific manner, we measured from the S6 chimera and the ShBΔ channels, two separate properties of channel gating: (1) the voltage-dependence of activation and (2) the maximum open channel probability. The voltage-dependence of activation was measured from the size of macroscopic tail currents following activation voltage pulses to various voltages. The voltage-dependence of the normalized tail current was then fitted to a Boltzmann distribution in order to determine the $V_{mid}$ (mV) and slope (b) (mV/e-fold change). From this fit, a slope parameter (b) and the midpoint potential (Vmid) are obtained. Values for ShBΔ: $V_{mid}$ = -45 ± 2 mV, slope = 3.7 ± 0.7 mV/e-fold, N = 8; Values for the S6 chimeric channel: $V_{mid}$ = -44 ± 2 mV, slope = 3.9 ± 0.4 mV/e-fold, N = 3 (mean ± SD). The tail currents at -100 mV were fitted with a single exponential using the program Clampfit (Axon Instruments). The time constants are not significantly different for ShBΔ: 3.4 ± 0.5 msec., N = 5, and the S6 chimera: 3.2 ± 0.7 msec., N = 4, mean ± SD.

The open channel probability was measured at +100 mV with each digitized point counted as either open or closed according to a 50% current threshold. $P_{open}$ for ShBΔ: 0.88 ± .03, N = 4; $P_{open}$ for the S6 chimeric channel: 0.92 ± 0.02, N = 4 (mean ± SD). The S6 transplantation did not alter the voltage-dependence or kinetic properties of channel gating, suggesting that the overall channel structure was not affected. Given that the differences in the H5 sequence between NGK2 and ShBΔ accounted largely for the differences in external TEA sensitivity but not single-channel conductance, it seems unlikely that the effect of the S6 transplantation on single-channel conductance could be attributed to an indirect effect via the H5 domain.

**Multiple Residues Spanning the S6 Domain Affect Single-Channel Conductance and Internal TEA and Barium Ion Sensitivity**

Eight amino acids are different between ShBΔ and the S6 chimera. To determine whether the alterations in pore properties arise from one or multiple amino acid differences in the S6 region, we analyzed three "mini-chimeras", I464L/L472M; S479N/N482G/Y483M; and

F484Y/H486S/R487L. The two triple mutations had single-channel conductance (Figure 3; Table 1) and internal barium sensitivity (E. Isacoff, personal communication) intermediate between those of ShBΔ and NGK2, but their internal TEA sensitivities remained very similar to that of ShBΔ (Table 1). In contrast, the double mutant had a single-channel conductance identical to that of ShBΔ though its internal TEA sensitivity was the same as that of the S6 chimera (Figure 3; Table 1). Of the two mutations included in the double mutant, the L472M mutation, but not the I464L mutation, had a reduced internal TEA sensitivity similar to that of the double mutant (Table 1). Among the six mutations included in the two triple mutants with a larger single-channel conductance, S479N and H486S increased the single-channel conductance (Figure 3; Table 1). Recent studies have shown that the mutation A463V alters the rate of slow inactivation and single-channel conductance (Hoshi et al., 1991) and mutations of T469 alter the sensitivity to internally applied quaternary ammonium blockers with extended alkyl chains without affecting the sensitivity to internal TEA (Choi et al., 1993). Thus, multiple residues spanning the S6 segment have been found to affect pore properties.

## DISCUSSION

Although previous studies have indicated that the H5 (also known as SS1-SS2 or P region; Guy and Conti, 1990; Hartmann et al., 1991) segment forms the pore structure (Yool and Schwarz, 1991; Hartmann et al., 1991; Yellen et al, 1991), we now feel that our results with mutations in the S6 domain and also with corresponding H5 chimeric mutations, help to localize the pore forming region and to identify individual amino acid residues responsible for TEA and barium ion binding. In addition, both the H5 and S6 segment appear to limit potassium conductance, perhaps by forming a tight constriction. The small conductance of DRK1 is probably due to its H5 segment restricting potassium flux, whereas the small conductance of ShBΔ appears to arise primarily from a limiting S6 segment. Only the H5 segment contains the external binding site for TEA.

The barium block of the S6 chimera was more similar to that of NGK2 than ShBΔ, such that the barium block showed a much greater voltage-dependence for NGK2 and the chimeric channel than for the ShBΔ channel.

Thus, barium ions appear to penetrate further into the pore of the NGK2 and chimeric channels, so that its binding to the channel pore is influenced by the electric potential across the membrane. This suggests that transplanting the S6-containing segment of NGK2 into ShBΔ has either introduced a barium binding site in the pore or alternatively has exposed such a binding site by reducing an energy barrier to barium entry. Barium ions are a much more sensitive probe of the pore structure of potassium channels as barium can mimic a potassium ion due to its similar size. Therefore, mutations affecting the binding, affinity or voltage-dependence of barium ion binding necessarily are directly involving the pore lining structure.

The leucine 472 to methionine mutation alone accounted for the effect of the S6 chimera on internal TEA sensitivity. Previous studies have shown that mutations of methionine 440 and threonine 441 in the H5 domain of *Shaker* also alter internal TEA sensitivity (Yellen et al., 1991; Choi et al., 1993). However, methionine 440 and threonine 441 are 100% conserved among all known voltage-gated potassium channel sequences (See Figure 5 in Chapter I) and therefore cannot account for the different internal TEA sensitivities of these channels. It is likely therefore, that the mutations in the H5 domain which affect internal TEA sensitivity are acting

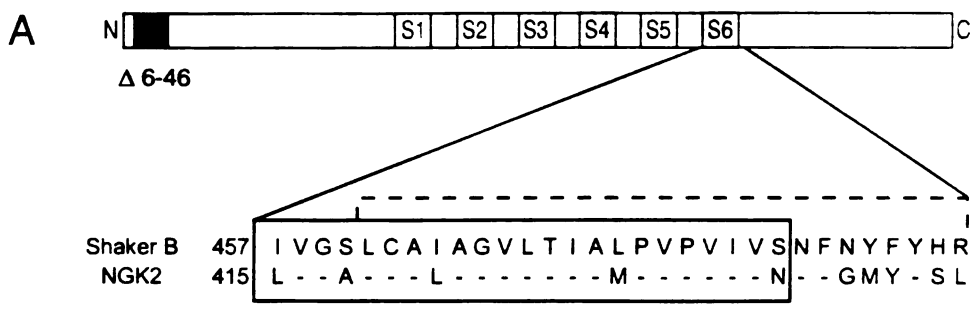
indirectly by influencing nearby amino acid residues which either form the internal TEA binding site or are located near it. In contrast to the results with the H5 mutations, a leucine is found at the position of 472 in the *Shaker* subfamily (See Figure 6 in Chapter I) whose members exhibit a high sensitivity to internal TEA (Kirsch et al., 1991; Taglialatela et al., 1991), while a methionine residue is found in the corresponding position in all known members of the Shaw subfamily including NGK2, which have a much lower sensitivity to internal TEA (Hartmann et al., 1991; Kirsch et al., 1991; Taglialatela et al., 1991). Thus the different internal TEA sensitivities of different potassium channels are likely to be due to a difference in the S6 segment at the position corresponding to leucine 472 in *Shaker B*.

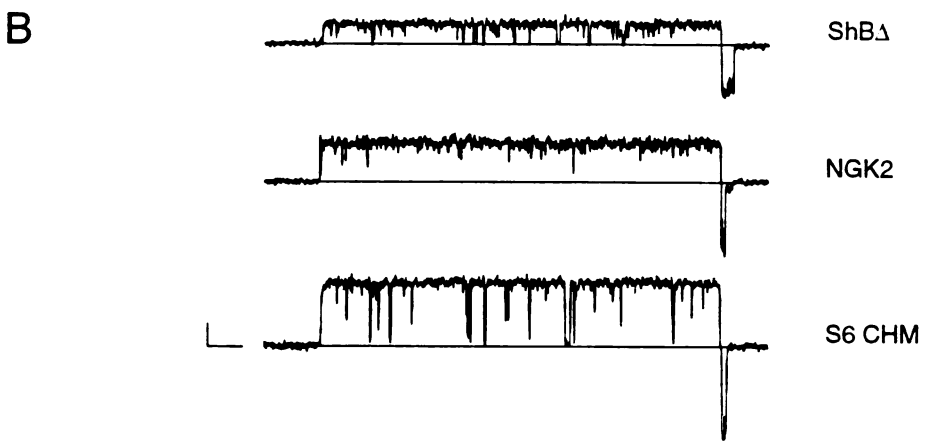
Since differences between NGK2 and ShBΔ in the blockade of the pore by cytoplasmically applied TEA and barium ions, as well as the permeation rate of potassium ions, can be accounted for by their differences in the S6 rather than the H5 sequence, our results strongly suggest that the pore of voltage-gated potassium channels is likely to be formed not only by the H5 sequence (Yool and Schwarz, 1991; Yellen et al., 1991; Hartmann et al., 1991; Kirsch et al., 1992; Heginbotham et al., 1992) and the S4-S5

intracellular loop (Isacoff et al., 1991; Slesinger et al., 1993), but also by the S6 domain.

## FIGURES AND TABLES

**FIGURE 1**


A
N
S1
S2
S3
S4
S5
S6
C
Δ 6-46
Shaker B 457 I V G S L C A I A G V L T I A L P V P V I V S N F N Y F Y H R
NGK2 415 L - - A - - - L - - - - - - - M - - - - - - N - - G M Y - S L


B
ShBΔ
NGK2
S6 CHM

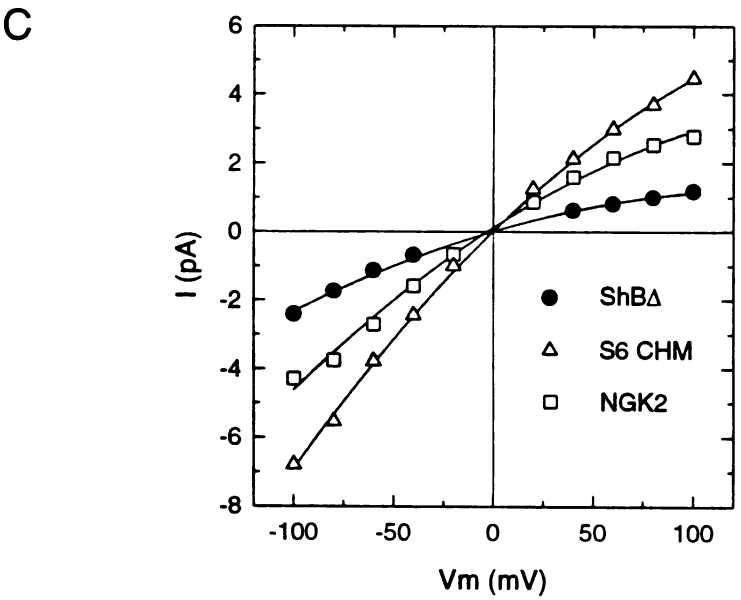
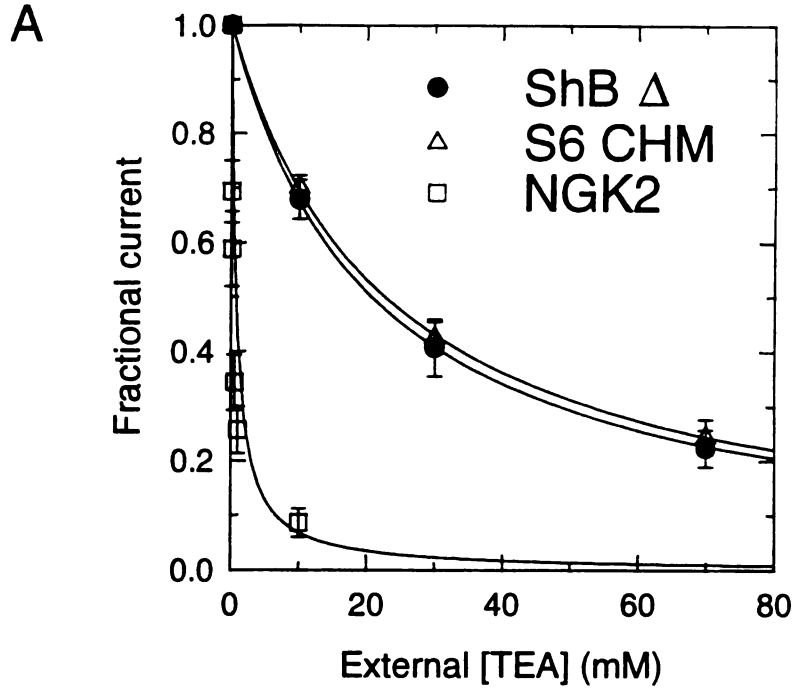

C
I (pA)
Vm (mV)
ShBΔ
S6 CHM
NGK2

**FIGURE 1 The ShBΔ-NGK2 S6 Chimeric Potassium Channel and Single-Channel Properties of ShBΔ, NGK2, and the S6 Chimera.**

**(A)** The amino acid alignment of the sixth proposed transmembrane segment (S6) and adjacent residues of the *Shaker B* potassium channel (Schwarz et al., 1988) and of the mammalian NGK2 channel (Yokoyama et al., 1989). The boxed region represents the proposed sixth transmembrane segment. The dashed bracket corresponds to the region which was transplanted from the NGK2 channel into ShBΔ. The dashes represent residues of NGK2 which are identical to the corresponding residues in ShBΔ. The numbers apply to the first residue in each of the aligned sequences. **(B)** Representative single-channel currents recorded from excised inside-out patches from *Xenopus* oocytes. **(C)** Representative open channel current-voltage relations. $V_m$, membrane potential; I, single-channel current. Scale bar: 1.4 pA, 30 msec.

**FIGURE 2**


A
1.0
0.8
0.6
0.4
0.2
0.0
Fractional current
ShB Δ
S6 CHM
NGK2
0
20
40
60
80
External [TEA] (mM)

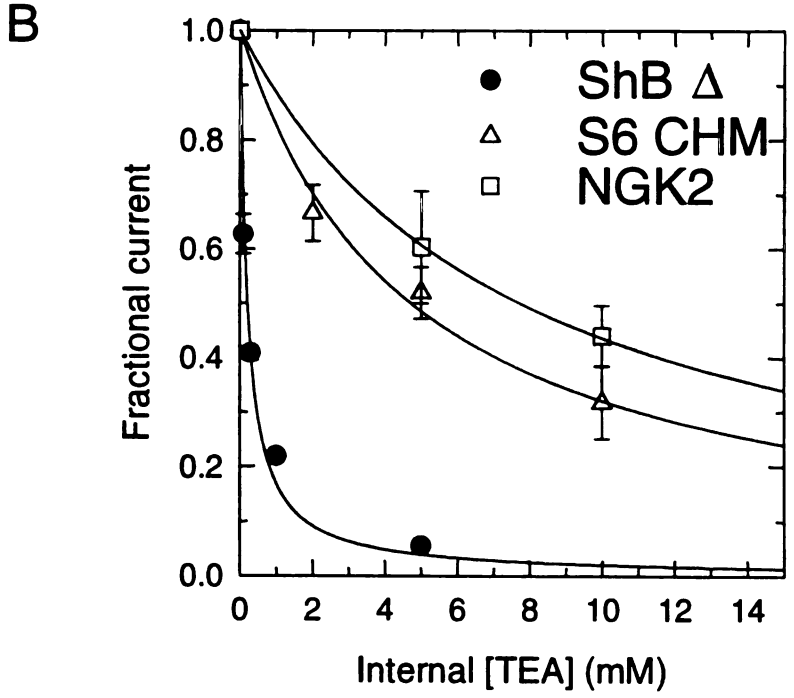

B
1.0
0.8
0.6
0.4
0.2
0.0
Fractional current
ShB Δ
S6 CHM
NGK2
0
2
4
6
8
10
12
14
Internal [TEA] (mM)

**FIGURE 2 Continued**

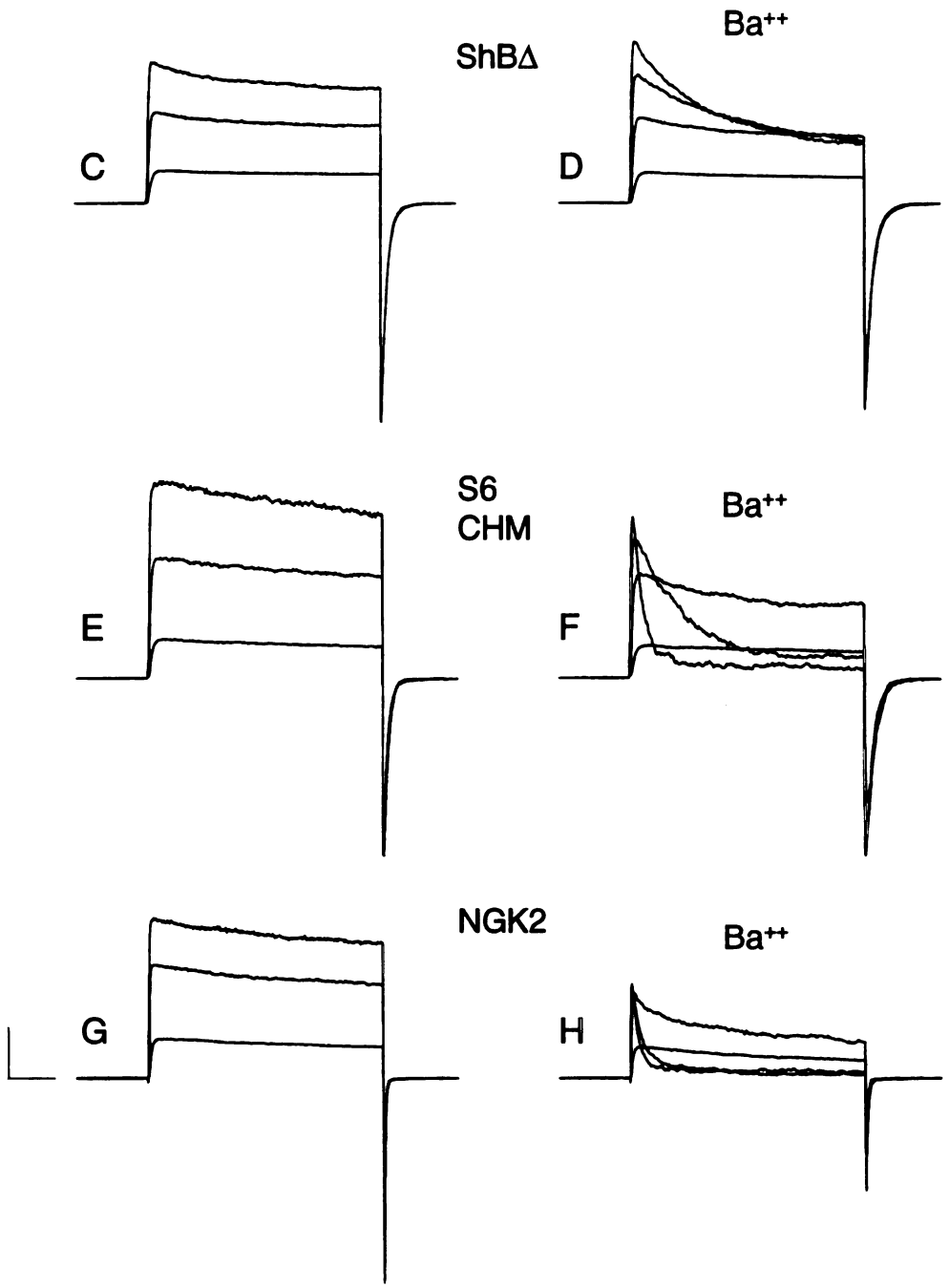


**FIGURE 2 Continued**

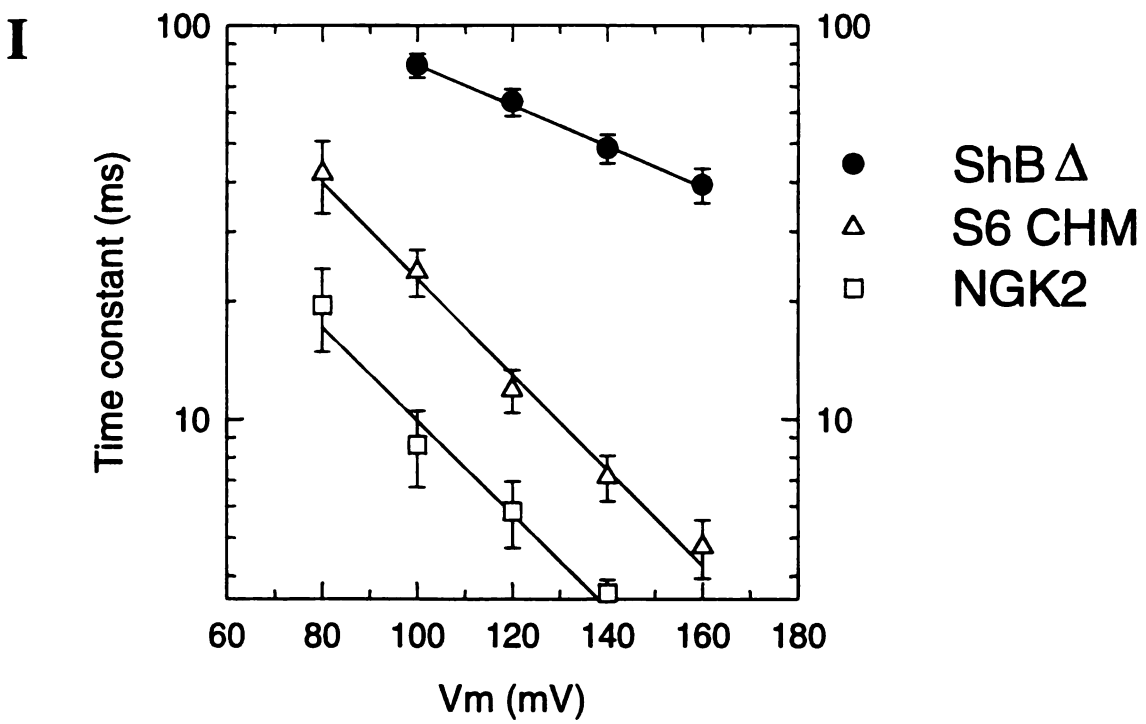


**FIGURE 2 TEA and Barium Ion Blockade of Currents from ShBΔ, NGK2, and the S6 Chimeric Channel.**

**(A)** Sensitivity to external TEA. The reduction in peak current at 0 mV for ShBΔ and the chimera or at +20 mV for NGK2, was measured as a function of external TEA concentration. **(B)** Sensitivity to internal TEA. The reduction in peak current at 0 mV was measured as a function of TEA concentration. Each point is the mean from at least six determinations and the error bars indicate the SD. The curves were fit to the equation:

$$\text{fractional Current} = 1 / ( 1 + [\text{TEA}] / K_i ).$$

**(C-I)** Sensitivity to internal barium ions. Ionic currents from ShBΔ **(C,D)**, the S6 chimera **(E,F)**, and NGK2 **(G,H)**, before (C,E,G) and after (D,F,H) application of internal barium ions. Excised inside-out patches were used with the recording solutions as follows: external (pipette) solution contained 98 mM KCl, 1 mM $MgCl_2$, 0.3 mM $CaCl_2$, 10 mM HEPES (pH 7.1, KOH). The internal (bath) solution contained 98 mM KCl, 0.5 mM $MgCl_2$, 1 mM EGTA, and 10 mM HEPES (pH 7.1, KOH) (20-23°C) (C,E,G), before perfusion with solution of the same composition except for the addition of 50 $\mu$M barium chloride (D,F,H). Holding potential was -100 mV with 160

msec depolarizing pulses from 20 mV to 100 mV in 40 mV increments for C, E, and G, or from 20 mV to 140 mV in 40 mV increments for D, F, and H. Scale bar: 980 pA, 32 msec. **(I)** Voltage-dependence of current block. The fast component of current block was fitted with a single exponential and the time constant was plotted as a function of membrane potential. The points are the means from at least three determinations and the error bars indicate SD. The line is a first order linear regression through the data points.

**FIGURE 3**

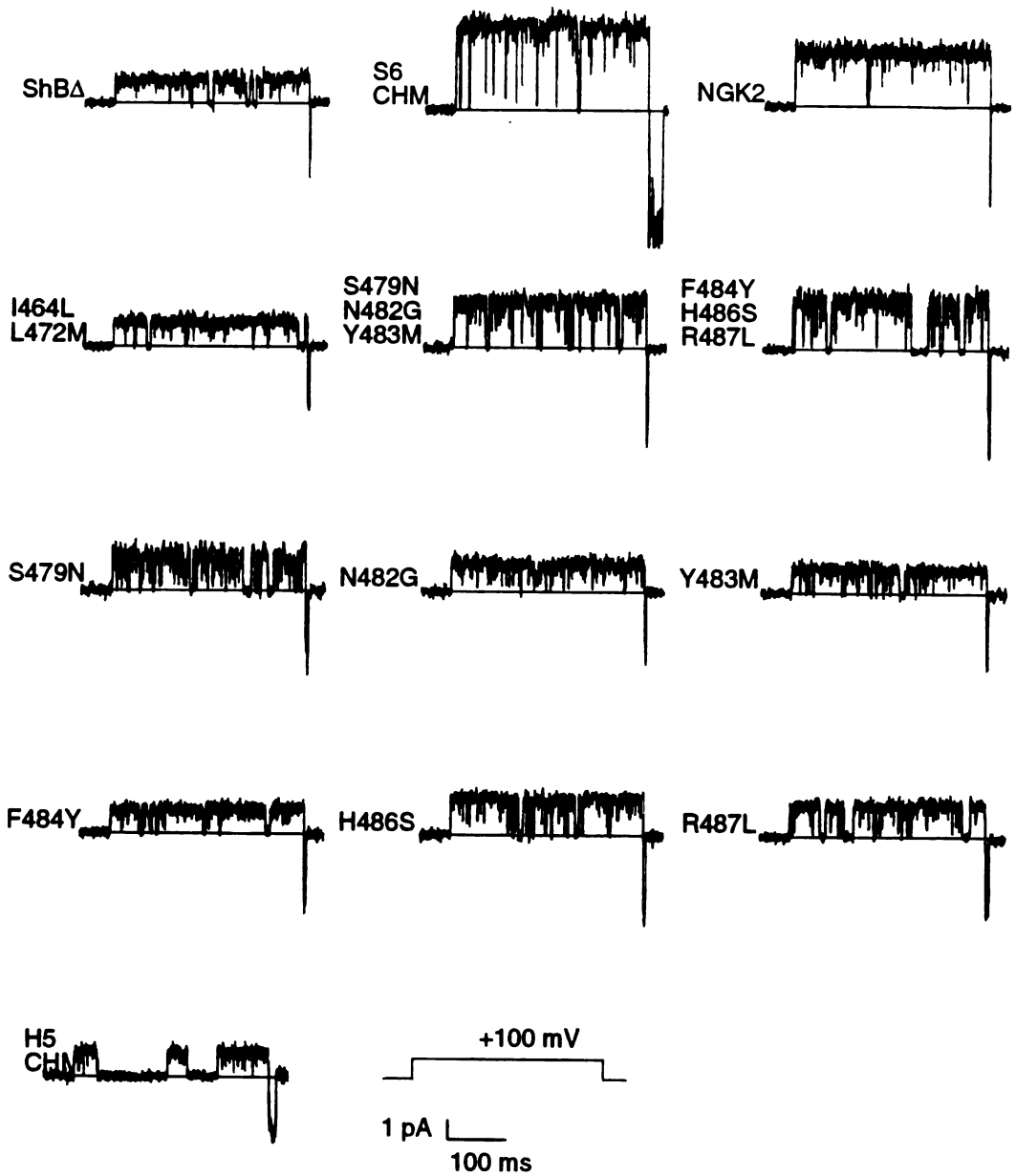

ShBΔ
S6
CHM
NGK2
I464L
L472M
S479N
N482G
Y483M
F484Y
H486S
R487L
S479N
N482G
Y483M
F484Y
H486S
R487L
H5
CHM
+100 mV
1 pA
100 ms

**FIGURE 3 Single-Channel Traces from the Wild-Type Channels ShBΔ and NGK2, the H5 Chimera, the Full S6 Chimera, the S6 "Mini-Chimeras" and the Individual Point Mutations in the S6 Domain.**

Representative single-channel currents evoked at +100 mv, recorded from excised inside-out patches from *Xenopus* oocytes. The recording solutions used were as follows: external (pipette) solution contained 98 mM KCl, 1 mM $MgCl_2$, 0.3 mM $CaCl_2$, 10 mM HEPES (pH 7.1, KOH). The internal (bath) solution contained 98 mM KCl, 0.5 mM $MgCl_2$, 1 mM EGTA, and 10 mM HEPES (pH 7.1, KOH) (20-23°C). Holding potential was -100 mV and the membrane was depolarized to +100 mV for 320 msec. Single-channel current amplitudes were measured from amplitude histograms and were leak subtracted using blank traces which contained no channel openings.

**TABLE 1 Single-Channel Conductances and Internal TEA Sensitivities from the Shaker Mutants.**

| Mutant | Chord Conductance (pS) | $K_i$ (mM) |
|---|---|---|
| | Mean ± SD (N) | Mean ± SD (N) |
| ShBΔ | 11.2 ± 1.0 (4) | 0.23 ± 0.06 (18) |
| S6 chimera | 43.8 ± 1.7 (5) | 5 ± 1.3 (17) |
| NGK2 | 27.0 ± 1.0 (7) | 8 ± 2.2 (6) |
| H5 chimera | 11.6 ± 0.3 (3) | nd† |
| I464L, L472M | 12.6 ± 0.8 (3) | 4.8 ± 1.4 (5) |
| S479N, N482G, Y483M | 20.5 ± 1.5 (3) | 0.34 ± 0.06 (3) |
| F484Y, H486S, R487L | 22.8 ± 1.1 (5) | 0.17 ± 0.04 (4) |
| S479N | 16.7 ± 0.6 (2) | nd |
| I464L | nd | 0.16 ± 0.02 (5) |
| L472M | nd | 3.1 ± 1.3 (4) |
| N482G | 14.4 ± 0.6 (3) | nd |
| Y483M | 12.6 ± 0.7 (4) | nd |
| F484Y | 11.2 ± 0.3 (3) | nd |
| H486S | 17.6 ± 1.2 (5) | nd |
| R487L | 13.7 ± 1.4 (4) | nd |

**TABLE 1 Single-Channel Conductances and Internal TEA Sensitivities from the Shaker Mutants.**

Experiments were done on excised inside-out patches from a holding potential of -100 mV. TEACl was applied by perfusing the intracellular side of the patch with a perfusion pipette brought into close proximity to the membrane. Excised inside-out patches were used with the recording solutions as follows: external (pipette) solution contained 98 mM KCl, 1 mM $MgCl_2$, 0.3 mM $CaCl_2$, 10 mM HEPES (pH 7.1, KOH). The internal (bath) solution contained 98 mM KCl, 0.5 mM $MgCl_2$, 1 mM EGTA, and 10 mM HEPES (pH 7.1, KOH) (20-23°C). $K_i$'s or half blocking concentrations for internally applied TEA at 0 mV were determined by fitting the fractional current remaining after an 80 msec voltage pulse to an equation that describes open channel block at a single site:

$$K_d(V) = K_d(0) \exp (\delta ZFV / RT)$$

where $\delta$ is the fraction of the electrical field traversed by the blocker in moving from the inside solution into the blocking site (Woodhull, 1973), except for ShBΔ, NGK2 and the S6 chimera which were determined from the TEA dose response curves from Figure 2. †Not determined due to its low

level of expression. nd: not determined. Amino acids: F, phenylalanine; G, glycine; H, histidine; I, isoleucine; L, leucine; M, methionine; N, asparagine; R, arginine; S, serine; Y, tyrosine.

# CHAPTER IV

# FUTURE DIRECTIONS AND CONCLUSIONS

In 1952 there was only one permeation pathway for potassium ions through the membrane. Depolarizing membrane voltages caused this conductance to activate with a sigmoidal time course possibly due to an interaction between charged or dipolar gating particles and the electric field. Whatever the actual mechanism was, only potassium flowed through this pathway. In the present day, we can interpret and expound on Hodgkin and Huxleys theoretical model and appreciate it even more given that they had no understanding of the physical nature of these ionic pathways. Today, we can dissect with molecular precision the genes responsible for ionic excitability and understand how an ion can interact with and flow through a protein imbedded in the membrane. In just the few years since the cloning of the first sodium and potassium channel genes, we have learned a great deal about the molecular mechanisms involved in the processes of channel activation, voltage sensing, inactivation, protein assembly, toxin binding, ion selectivity, and ionic blockade and permeation. The number of references cited in this thesis attests to this remarkable accomplishment with more on the way.

In addition to the results described in the previous three chapters, I have also uncovered other intriguing avenues for future research on the *Shaker* voltage-gated potassium channel. For example, the receptor for the inactivation gate has only preliminarily been investigated by mutations of the S4-S5 domain (Isacoff et al., 1991). I have found in preliminary experiments that the S6 domain and residues immediately downstream also make up part of the receptor for the inactivation gate. Single point mutations in this region slow the rate of fast inactivation similar to the effects of deletional mutations in the amino terminus. This result along with the experiments in the S4-S5 domain, demonstrate that the inner mouth and vestibule of the channel is constructed from widely spaced domains of the channel protein and that the receptor for the inactivation ball is at the entrance to the pore. More work needs to be done in order to localize and characterize at the amino acid level the receptor, but now I have narrowed down the region where we can begin the exploration.

The study of ionic channels has tremendous importance not only for the basic scientists studying membrane proteins but also for the clinicians who study diseases which we are now only beginning to understand at the molecular and cellular level. For example, it has recently been discovered that a group of human neuromuscular diseases,

hyperkalemic periodic paralysis and paramyotonia congenita, are due to point mutations in one of the sodium channel genes expressed in muscle (Cannon et al., 1991; Ebers et al., 1991; Ptacek et al., 1991; Ptacek et al., 1992; Ptacek et al., 1993; Cummins et al., 1993). Interestingly, some of the mutations which I created in the laboratory mimic in effect several of the mutations discovered in humans. In addition to these neuromuscular diseases, the study of ion channel biophysics also have implications in other human diseases which are now known to be due to molecular defects in ion channels such as in cystic fibrosis. Cystic fibrosis is a heterogenous disease affecting mainly children and young adults. The molecular defect lies in point mutations in a chloride channel found mainly in pulmonary epithelial cells and in exocrine glands. The disease is heterogenous in phenotype because there exists a multitude of different mutations in the gene (Welsh and Smith, 1993). Again as with the disease mentioned above, the study of ion channels has shed light on the physiology of the altered protein. Recently, a neurological disease, episodic ataxia, has been identified whose cause is due to a mutation in a voltage-gated potassium channel (Litt et al., 1994). There awaits other diseases which have yet to be linked to potassium channel mutations (Etcheberrigaray et al., 1993).

For other human disorders, the study of potassium channels has great

potential for such disease as hypertension, angina, cardiac arrhythmias, asthma, bladder instability, and multiple sclerosis (Cook, 1988; Andersson, 1992; Quast, 1992; Bohuslavizki et al., 1992; Weston and Edwards, 1992; Kato, 1993; McPherson, 1993; Cavero and Premmereur, 1994). With the combined use of electrophysiology and mutational analysis we should in the near future be able to design specific pharmacological agents for tissue and disease specific potassium channels.

Even with the information derived from mutational analysis and electrophysiology, we still do not have a three dimensional structure for the potassium channel. This is ironic given that the potassium channel protein is one of the most mutagenized proteins studied to date. At present, the structure of only one membrane protein has been solved at atomic resolution (the resolution necessary for understanding ionic permeation), the photosynthetic reaction center from bacteria. Hopefully, the potassium channel's turn will come. We await this moment eagerly ...

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