STRUCTURE-FUNCTION STUDIES OF DROSOPHILA *SHAKER* POTASSIUM CHANNELS

Thesis by
Ken McCormack

In Partial Fullfillment of the Requirements
for the Degree of
Doctor of Philosophy

California Institute of Technology
Pasadena, California

1991
(Submitted March 7, 1991)

ACKNOWLEDGEMENTS

I would like to acknowledge members of the Tanouye, Rudy and Iverson laboratories for their contributions to the work presented in this thesis. First, I would like to mention my advisor, Mark Tanouye, who allowed me to pursue experiments which I found interesting; his disposition was greatly appreciated. I am also indebted to those who personally instructed me in the techniques necessary to develop questions into sometimes interpretable data. Mani Ramaswami, Linda Iverson and Sasha Kamb were proficient instructors who made themselves available to me-friends you can easily take for granted. Bernardo Rudy was of enormous help and I admire him most for his ability to juggle and argue at the same time. Jim Campanelli's short tutorial on practical electrophysiology would have been sorely missed. I would also like to thank Henry Lester and other members of his lab for advice and oocytes, and Norman Davidson and Doug Rees for support and discussions of the work described in Chapter 2B.

On short notice I received helpful comments on the Introduction, Discussion and Appendix of this thesis from my friends, Rich Mooney, Zaven Kapriellian and Tom McCormack. Thanks also go to Tobi and Manny Delbruck for making my life much easier this past month.

ABSTRACT

Voltage-dependent ion channels mediate electrical signals in the nervous system; many sodium (Na+), calcium (Ca++) and potassium (K+) selective channels are structurally related, and thus represent a family. These proteins undergo interesting conformational changes in response to alterations in transmembrane potential. However, the functional determinants involved in these transitions are not well understood. Chapters 2A and 2B describe the identification and characterization of an amino acid sequence motif (a leucine-heptad repeat) that is evolutionarily conserved among this family of voltage-dependent ion channels. Conservative, single amino-acid substitutions within this region of *Drosophila Shaker* (*Sh*) proteins have substantial effects on the voltage-dependence of activation. The observed alterations suggest that the heptad-repeat region is an important determinant in the conformational transitions leading to channel opening.

Na+ and Ca++ channels are composed of four homologous domains, each of which is equivalent to a single K+ channel subunit. Thus, K+ channels are thought to be functional multimers. Furthermore, there are a large number of different voltage-dependent K+ genes and alternatively spliced products that potentially can be expressed in the same cell. Therefore, the potential number of different K+ channel multimers could be quite extensive. Chapter 3 describes the physiological characteristics of combinations of K+ channels belonging to the *Sh* family that have been co-expressed in *Xenopus* oocytes. Members of the same molecular class of *Sh*

channel form heteromultimers with novel functional properties, adding to the diversity of K+ channel function. Members of different molecular classes do not form heteromultimeric channels, suggesting that there are distinct K+ channel systems. The Appendix describes an alternative exon in the "constant" region of the *Drosophila Sh* gene, the existence of which suggests, that the molecular diversity of this gene is greater than previously determined.

TABLE OF CONTENTS

Chapter 1

Introduction

Biological organisms have evolved greater degrees of complexity and organization in the midst of an entropic universe. This was made possible by the compartmentalization of biological processes. Cellular and intracellular compartments provide a means for establishing "nonequilibrium systems," which can acquire a net input of energy and thus, sustain cellular processes, as well as develop higher levels of complexity through evolution. The processes mediating the energetics and interactions of "cells" with their environment, such as transport and channel processes, are closely linked to compartmentalization.

In one of the most spectacular evolutionary adaptations, the nervous and neuromuscular systems found in higher organisms, ion channel proteins have evolved diverse and specialized functions. In these tissues ion channel proteins are directly responsible for the primary function of cells within the nervous system: electrical signalling. Action potentials are electrical signals which are propagated along individual cells through the activation of voltage-dependent ion channels. Signalling between cells generally takes place at specialized sites called synapses, which can either be electrotonic, where ionic currents spread passively from one cell to another through the pores of gap-junction proteins, or chemical, where a presynaptic cell releases transmitter ligand-molecules that diffuse to the postsynaptic cell, bind to a receptor-channel and thereby activate it. Activated ion channels can selectively change the ionic permeability of membranes, thus altering the membrane resistance or the transmembrane potential. Membrane excitability is dependent on the electrochemical gradients of ions (discussed below); the ionic selectivity of channels determines whether their opening is inhibitory or excitatory.

The large number of channel types and subtypes provides a wide variety of electrical properties in excitable cells. Potassium (K+) channels form the most diverse group of channel proteins; the type of K+ channel expressed in a given cell can play a crucial role in determining its waveforms and firing frequencies (1-3). Thus, channel proteins can influence the output properties of cellular networks within the brain. The modulation of channel activity is directly involved in determining some forms of learning and memory (4-6). Therefore, channel proteins provide the most likely candidate for a molecular basis of subjective experience. The work presented here has focused on some of the structure-function relationships of the *Drosophila Shaker* (*Sh*) gene product, a voltage-dependent K+ selective channel, in an attempt to understand the mechanisms by which it operates.

Physiology of voltage-dependent ion channels

Although the electrical properties of the nervous system were discovered in 1791 by L. Galvani, it was only with the recent technical advances, such as intracellular recordings and voltage-clamp techniques that the foundations of modern electrophysiology were established. Intracellular recordings in the squid giant axon revealed a transmembrane electrical potential. Furthermore, depolarization of the membrane above a certain threshold activated an all or none propagating excitation of the membrane which was termed the "action potential" (7). A. Hodgkin and A. Huxley then established that selective and independent changes in ionic permeabilities within the axon membrane were responsible for the action potential. With the use of voltage-clamp techniques, in combination with ionic substitution,

they examined the ionic basis of the action potential (8), which is summarized as follows:

Electrochemical basis of ionic currents. Although the diffusion of uncharged particles down a concentration gradient is a random, entropy-driven process (9), ions, because of their charged character, generate an electrical driving force or equilibrium potential when distributed unequally. The relationship between the concentration of a charged particle on one side of the membrane with respect to the other and the potential is expressed by the Nernst equation:

$$E_I = RT/_{nF} \ln [I]_o/[I]_i$$

E is the ionic equilibrium potential in Volts, R is the universal gas constant in Joules/mole-K, T is the temperature in degrees Kelvin, n is the valence of the ion, F is Faraday's constant in coloumbs/mole and [I] represents the concentration of an ion (o) partitioned from another concentration of the same ion (i).

The net flow of charged particles produces a current (I) which is determined by Ohm's law :

$$V = IR \quad \text{or} \quad I = gV$$

I is the current in amperes, V is the amount of volts, R is the resistance in ohms and g is the conductance, the reciprocal of R, measured in siemens.

Several ions determine the potentials of cellular membranes. The concentrations of these ions and their relative permeabilities determine the membrane potential (V_m): ions with the largest relative permeabilities drive

the membrane potential toward their equilibrium potential and therefore dominate the potential difference across the membrane. Using instantaneous current-voltage relations (by altering V_m during the permeability increase) Hodgkin and Huxley determined that the ionic permeabilities depended on membrane potential and not membrane current, and that they were Ohmic; thus, the permeabilities could be considered conductances in coordination with the electrochemical or Nernst potentials of the respective ions (10, however, see also ref. 1). The currents generated by each ion were based on the net driving force, i.e., the difference between the respective ionic Nernst potential and V_m. The currents for the major ionic species, Na+, K+ and Cl-, are given by:

$$I_{Na} = g_{Na}(V_m - E_{Na}) \qquad I_k = g_k(V_m - E_k) \qquad I_{Cl} = g_{Cl}(V_m - E_{Cl})$$

$I_{Na,k,Cl}$ are the currents in amperes, $g_{Na,k,Cl}$ are the conductances in siemens and $E_{Na,k,Cl}$ represent the Nernst equilibrium potential for the respective ions. V_m is the membrane potential in volts. The parameter $(V_m - E_{Na,k,Cl})$ provides the driving force for current flow of the respective ion.

At rest, there are differences in ionic permeabilities across cellular membranes. K+ ions are generally more permeable and therefore V_m is close to E_k. However, no net current flows across the membrane at rest, and therefore the sum of the ionic currents is zero. Solving for V_m at rest, using the sum of the ionic currents, obtains:

$$V_m = \frac{E_k + (g_{Na}/g_k)E_{Na} + (g_{Cl}/g_k)E_{Cl}}{1 + (g_{Na}/g_k) + (g_{Cl}/g_k)}$$

Experiments by Hodgkin and Huxley showed that upon depolarization above a certain threshold, Na+ permeability suddenly increased, driving the membrane potential toward the equilibrium potential for Na+ (about +60 mV). This Na+ permeability inactivated in a time-dependent manner, while a second slower phase of K+ permeability activated, and the driving force of K+ (E_k is approximately -70 mV) caused the membrane potential to return to rest (about -60 mV), sometimes after a brief overshoot (8). Thus, the membrane excitability properties which underlie action potentials result from permeability increases to specific ions.

Hodgkin and Huxley went on to develop equations that modeled the independent currents and conductance-voltage relationships of Na+ and K+ in order to determine whether a combination of the individual conductances was sufficient to develop action potentials (11). The kinetics of Na+ currents and conductances were best fit by a third order dependence on a voltage-dependent rate constant (three independent gating particles, m^3) for activation, and a single voltage-dependent rate constant for inactivation (an independent inactivating particle, h), i.e., m^3h kinetics. K+ currents (which did not inactivate in squid axon) were best fit by a fourth order dependence on a voltage-dependent rate constant or n^4 kinetics (see refs. 11 or 1 for further review).

With few exceptions the form of these equations and rate constants accurately mimicked the squid giant axon action potentials, as well as the individual ionic conductances and currents. However, some exceptions are noted below. In addition, Hodgkin and Huxley suggested that a "gating current," due to the rearrangement of the voltage-sensing particles (3 for Na+ and 4 for K+) during the activation of the permeating molecules (channels),

should produce a small but measurable current (11), although they themselves were unable to obtain evidence for any such current:

"The dependence of g_{Na} and g_K on membrane potential suggests that the permeability changes arise from the effect of the electric field on the distribution or orientation of molecules with a charge or dipole moment."

Approximately twenty years later, in the early 1970s, gating currents associated with Na+ channel activation were measured by several groups (12-14). These gating currents have been examined in several preparations and a comparison of their properties with those predicted by the Hodgkin & Huxley model have been reviewed extensively; for Na+ channels neither the voltage-dependence of the charge movement nor the time constants of the on and off gating currents directly reflect the activation variable *m* (15-17). Furthermore, in contrast to the Hodgkin and Huxley model, the inactivation of Na+ channels does not appear to be substantially voltage-dependent, nor does it appear to be independent from the activation process (18). K+ channel gating currents have been examined less extensively (19, 20).

Just prior to the beginning of the last decade another technical advance in physiology, the patch clamp, was developed by E. Neher and B. Sakmann. Through high resistance electrode-membrane seals (10^{11}ohms), ionic currents through a small number of channels or even single channels could be resolved (21, 22). The ability to examine the catalytic activity of a single protein (from ionic currents) is unique. *Sh* and other voltage-dependent ion channels are now amenable to patch clamp techniques in native tissues and, through the advances in the molecular biology of these channels, heterologous expression systems. The use of heterologous expression systems,

such as *Xenopus* oocytes, enables the isolation of particular channel types for characterization; these systems are particularly well suited for the analysis of structure-function relationships in combination with molecular techniques such as site-directed mutagenesis.

The diversity of channel proteins

The transmission of electrical signals is regulated by several different ion channel types that utilize ionic electrochemical gradients (as shown by Hodgkin and Huxley for voltage-dependent ion channels). Historically, channels have been classified into three categories: gap-junction, ligand gated and voltage-dependent ion channels. Electron micrographs have shown that gap-junction (23), nicotinic acetylcholine (Ach) receptors (ligand-gated channels) (24) and Ca++ release channels (25) are pseudo-symmetric multimeric aggregates with central pore-forming regions. Although no structural data exists for voltage-dependent ion channels they are also thought to form pores.

For channel proteins, the number of subunits or structural units that line the pore partially determines the selectivity of the permeating molecules (26). Voltage-dependent ion channels are the most selective channels. They conduct specific ions (for which they are named) and probably contain only four structural units surrounding the pore; the alpha- subunit of Na+ (27) and Ca++ (28) channels contain 4 pseudo-subuints or homology domains, while K+ channels are probably tetrameric aggregates of individual subunits (29, 30, see Fig. 1). Gap-junction proteins form large pores through the aggregation of six subunits. These pores allow the permeation of ions and

other larger molecules. Ach receptors, the best characterized ligand-gated channel, aggregate into pentamers which show intermediate selectivity; they are permeable to most cations.

In addition to these channel proteins, there are many others for which less structural data is known. Ligand-gated channels are named after the ligand which activates them. There are ligand-gated channels ($GABA_A$ and GlyR) which show homology to Ach receptors, but are selective to anions (31), and channels linked to ligand-gated receptors by second messengers (32). However, these classifications are based on physiological characterization that is sometimes misleading with respect to evolutionary relatedness. For example, a voltage-dependent chloride channel (33) and a voltage-dependent K+ channel (34) show no evolutionary relatedness to a superfamily of voltage-dependent channel proteins, while a cGMP activated channel (35) which shows little voltage-dependence (36, 37), and Ca++-activated K+ channels(38) do appear to belong to this superfamily. The work of this thesis focuses on structure-function studies of *Sh*, a voltage-dependent K+ selective channel which belongs to the superfamily of voltage-dependent ion channels (29, 30).

Molecular Biology of Voltage-Dependent Ion Channels

Sodium Channels

The first voltage-dependent ion channel gene to be isolated was the Na+ channel from *Electrophorus electricus* electroplax. This tissue is richly endowed with Na+ channels, permitting biochemical purification and peptide microsequencing (27). Subsequently, a small family of Na+ channels

have been cloned in rat and other species. The deduced amino acid sequences of the Na+ channel alpha subunit (some tissues contain one or two other subunits which primarily affect inactivation) indicates characteristic structural features for these channels (39-42). The proteins are large polypeptides of about 1800 amino acids with cytoplasmic amino (N) and carboxyl (C)termini. These proteins contain 4 individual subdomains (or homology domains), which are similar to each other; each domain is probably composed of 6 transmembrane segments (designated S1-S6) one of which may be the S4 domain. The S4 domain, has a characteristic motif of Arg-X-X reiterated from 4-8 times (where Arg is sometimes replaced by Lys, X is any hydrophobic residue and homology domains differ in the number of reiterations of the motif from 4-8 times). This domain, because of its motif of charged and hydrophobic residues, has been suggested to be a transmembrane segment which acts as a transmembrane voltage-sensor for the channel (39-42), although originally it was proposed to be a cytoplasmic domain (27).

Calcium Channels

The first Ca++ channel was cloned by virtue of its affinity for dihydropyridines (28). The dhp-receptor, or alpha-subunit of the L-type Ca++ channel (Ca++ channels contain several other subunits), shows striking homology to Na+ channels: 4 homology domains containing 6 probable transmembrane segments including an S4 domain and cytoplasmic N and C terminal ends. Physiological evidence suggests a larger diversity of Ca++ channel types than Na+ channel types; thus, there may be more significant variations in the structures of the alpha subunits of these channels. However, it is also possible that some of the physiological diversity of these channels may be a function of the associated subunits.

Molecular diversity of K+ channels

Voltage-dependent K+ selective channels were probably the first ion-selective channel to evolve; in comparison to other types of voltage-dependent ion channels they comprise an extremely diverse family (1,3). In general, the K+ channel types expressed in a given cell largely determines its membrane potential, action potential waveforms and firing patterns (2, 3, 43). Some of the channel types that have been characterized physiologically are listed in Table 1. Unlike Na+ and Ca++ channels, no high-affinity pharmacological ligands were available for the isolation and cloning of K+ channels until quite recently. Instead, molecular-genetic methods were used to isolate the first K+ channel gene, the *Drosophila Shaker* (*Sh*) gene.

Initially, *Sh* was identified as a *Drosophila* mutation which resulted in a characteristic leg-shaking phenotype by Catsch in 1944; subsequently more than 25 other alleles of *Sh* have been identified. The phenotypic defect of *Sh* alleles was suggested to reflect alterations in K+ currents by several lines of experimental evidence (44-46). However, the most compelling evidence that the *Sh* gene encoded the structural gene for a voltage-dependent K+ channel, came from voltage-clamp analysis of larval and pupal muscle. These experiments showed that only an inactivating or transient "A" type current, among several types of currents seen, was absent in *Sh* mutants. Furthermore, in the Sh^5 mutation, alterations in the voltage-dependence of activation and inactivation, as well as the kinetics of inactivation were observed in the "A" type current (47, 48).

The molecular cloning of the *Shaker* gene in *Drosophila* was reported simultaneously by several groups (49-51). Deduced amino acid comparisons of *Sh* showed that it was homologous to Na+ and Ca++ channels. However, it

also differed in that *Sh* products were equivalent to a single homology domain found in the other channels (Fig. 1). Analysis of the deduced amino acid sequences revealed 6 probable transmembrane domains, including an S4 domain. Sequence homologies suggest that Na+, Ca++ and K+ channels belong to the same superfamily, and that *Sh* channels are functional tetramers (29, 30). The molecular organization of the *Sh* gene is complex; *Sh* spans over 120 kilobases of genomic DNA and contains over 20 exons. Furthermore, *Sh* codes for several distinct products generated by alternative splicing of both the 5′ (or N-terminal) and 3′ (or C-terminal) ends (52-54). All full length *Sh* cDNAs obtained contain a constant central region onto which these alternative ends are spliced. However, there is some controversy as to the extent of this constant region, and in the Appendix of this thesis a putative alternative exon, coding for the last exon of the constant region proposed by Schwarz, et al., (54) is described.

More recently, low-stringency hybridization and Polymerase Chain Reaction (PCR) techniques have been used to isolate *Sh* homologs in other invertebrate species, such as Aplysia (55, Campanelli and McCormack unpublished results), and vertebrate species, including mouse (56), rat (57) and humans (58). In both of the invertebrate species multiple *Sh* gene products are generated through alternative splicing (52-55). In contrast, in mammalian species, there are several independent *Sh* genes which do not appear to be alternatively spliced (58-60). Thus, different mechanisms are utilized in vertebrates and invertebrates for the generation of diversity in the *Sh* class of K+ channel.

Other K+ channel genes were isolated in *Drosophila* with *Sh* probes through low-stringency hybridization; *Shab*, *Shaw* and *Shal* (61). The deduced amino acid sequences of these genes are homologous to *Sh* : there are six

predicted transmembrane domains including an S4 domain. There is evidence that some of these genes are alternatively spliced (62), although probably not as extensively as *Sh*. These genes indicate a family of *Sh* type K+ channels in *Drosophila*. Homologs of *Shab* (63) and *Shaw* (64) and *Shal* (61) have been cloned in rat, and for at least one of these classes (65), an extensive family has been observed. Thus, the diversity of K+ currents observed electrophysiologically is in large part due to the number of K+ genes and/or alternatively spliced products.

Recently, sequences have been reported for other *Drosophila* genes: *slowpoke* and *ether a go-go*. Mutations in these genes affect Ca++ activated and voltage-dependent K+ channels not previously isolated; these sequences appear to define new classes of K+ channel genes which are more distantly related to *Sh* (38). Several types of K+ channels, which have been characterized electrophysiologically, have not been isolated thus far and it is possible that these other types of K+ channel genes have yet to be cloned, e.g, inward-rectifiers, ATP-gated, G-protein gated and "M" channels. Alternatively, it is possible, although somewhat more unlikely, that combinations of genes previously isolated may form heteromultimeric channels with these properties (see Chapter 3). The diversity of K+ selective channel genes may be extensive.

Physiological Properties of *Sh* channels

The idea that the *Sh* gene coded for the structural element of a voltage-dependent K+ channel was substantiated by its amino acid sequence homology with Na+ and Ca++ channels. It was shown definitively by the

production of voltage-dependent K+ selective currents in *Xenopus* oocytes upon injection of *in-vitro* synthesized *Sh* RNA (66, 67); functional channels were obtained through injection of RNA coding for the translation of single *Sh* products. Moreover, the alternatively spliced *Sh* products produced currents that varied in the extent of inactivation and recovery from inactivation (68, 69).

Pharmacology of *Sh* products. The currents generated by *Drosophila Sh* products in *Xenopus* oocytes are similarly sensitive to block by 4-Aminopyridine (4-AP), Charybdotoxin (CTX) and tetraethylammonium (TEA) suggesting that these agents interact with structures in the constant region (see above or Appendix) of these channels. The block of K+ channels by 4-AP remains somewhat controversial; it may act on different K+ channels through different mechanisms. Although not studied definitively in *Sh* channels, in general it appears that 4-AP acts to block K+ currents in two ways: 1) by binding to the closed state of the channel and inhibiting it's conformational transition to an open state (70, 71), or 2) by blocking the conductance of the open state (72). The major effect of 4-AP observed in a single channel study on transient "A" type K+ currents in dorsal root ganglion cells from adult guinea pigs were due to changes in close-open kinetics, while some changes were seen in single channel conductance at higher concentrations of 4-AP (73). *Sh* products are completely blocked at approximately 3 mM 4-AP. Mutations of *Sh* affecting the block of 4-AP are described in Chapter 2B (74).

TEA is a charged quanternary ammonium ion, which depending on the type of K+ current, blocks K+ conductance from either the inside and/or the outside of the channel pore (1). The idea is that its large size and ionic

nature allow it to partially enter the pore where it becomes bound and blocks K+ permeation. *Sh* channels are not particularly well blocked by TEA: concentrations of as high as 50 mM do not completely inhibit *Sh* currents. However, the block by TEA is widespread throughout the range of K+ channel types, suggesting that the conducting pores of these channels share common structural features. Furthermore, TEA has been used in combination with CTX to determine a portion of *Sh* channels which influences conductance properties (see Chapter 4).

CTX is a polypeptide (4.3 kd) derived from scorpion toxin which blocks Ca++ activated and *Sh* (in *Xenopus* oocytes) K+ currents with high-affinity (K_d = 3.7 nM for *Sh*). In Ca++ activated K+ channels CTX and TEA compete to block the channel. Furthermore, the block by CTX is subject to electrostatic effects, i.e., the ionic strength of the external medium. Since TEA is believed to block near the external mouth of the channel pore in these channels, CTX has been suggested to block at an external site near the pore as well (75).

The molecular cloning of *Sh* has enabled the isolated examination of its properties in heterologous expression systems, such as *Xenopus* oocytes. Furthermore, through molecular manipulations, such as site-directed mutagenesis, it has become possible to examine specific structure-function relationships of *Sh* and other *Sh* family members. In this thesis, the investigation of several-structure function relationships in *Sh* are described. In Chapters 2A and 2B the identification and characterization of the leucine-heptad repeat region, conserved among most voltage-dependent ion channels, is presented. This work addresses the mechanisms of voltage-dependent gating and subunit assembly in *Sh* channels. In Chapter 3 an investigation of the properties of heteromultimeric channels is described. The results of this study further relate to association of subunits in *Sh* and other

Sh family members, and to the diversity of K+ channels expression *in vivo*. The Appendix presents the characterization of an alternative exon in the constant region of the *Drosophila Sh* gene; the molecular diversity of this gene is more complex than previously thought. Finally, Chapter 4 summarizes the recent literature on structure-function relationships of voltage-dependent ion channels.

References

1. Hille, B. "Ionic Channels of Excitable Membranes." (Sinauer, Sunderland, MA). (1984)

2. Llinas, R. "The intrinsic electrophysiological properties of mammalian neurons: insights into central nervous system function." *Science* **242**, 1654 (1988)

3. Rudy, B. "The diversity and ubiquity of K channels." *Neuroscience* **25**, 729 (1988)

4. Kandel, E.R. & Schwartz, J.H. "Molecular biology of learning: modulation of transmitter release." *Science* **218**, 433 (1982)

5. Alkon, D.L. "Voltage-dependent calcium and potassium conductances: a contingency mechanism for an associative learning model." *Science* **205**, 810 (1979)

6. Morielli, A.R., Matera, E., Kovac, M.P., Shrum, R., McCormack, K. & Davis, W.J. "Cholinergic suppression: a postsynaptic mechanism of long-term associative learning." *Proc. natn. Acad. Sci. USA* **83**, 4556 (1986)

7. Hodgkin, A.L., Huxley, A.F. & Katz, B. "Measurement of current-voltage relations in the membrane of the squid giant axon of *Loligo*." *J. Physiol.* **116**, 424 (1952)

8. Hodgkin, A.L. & Huxley, A.F. "Currents carried by sodium and potassium ions through the membrane of the giant axon of *Loligo*." *J. Physiol.* **116**, 449 (1952)

9. Einstein, A. "Investigations on the Theory of the Brownian Movement." (Dover, New York, NY) (1956)

10. Hodgkin, A.L. & Huxley, A.F. "The components of membrane conductance in the giant axon of *Loligo*." *J. Physiol.* **116**, 473 (1952)

11. Hodgkin, A.L. & Huxley, A.F. "A quantitative description of membrane current and its application to conduction and excitation in nerve." *J. Physiol.* **116**, 500 (1952)

12. Armstrong, C.M. & Benzinilla F. "Charge movement associated with the opening and closing of the activation gates of the Na channels." *J. Gen. Physiol.* **74**, 691 (1974)

13. Keynes, R.D. & Rojas, E. "Kinetics and steady-state properties of the charged system controlling sodium conductance in the squid giant axon." *J. Physiol.* **239**, 393 (1974)

14. Meves, H. "The effect of holding potential on the asymmetry currents in squid giant axons." *J. Physiol.* **243**, 847 (1974)

15. Armstrong, C.M. "Sodim channels and gating currents." *Physiol. Rev.* **61**, 644 (1981)

16. French, R.J. & Horn, R. "Sodium channel gating: models, mimics and modifiers." *Ann. Rev. Biophys. Bioeng.* **12**, 319 (1983)

17. Meves, H. "Hodgkin-Huxley: thirty years after." *Curr. Topics Membr. Trans.* **22**, 279 (1984)

18. Armstrong, C.M. & Benzinilla F. "Inactivation of the sodium channel. II. Gating current experiments." *J. Gen. Phys.* **70**, 567 (1977)

19. Benzinilla, F., White, M.M. & Taylor, R.E. "Gating currents associated with potassium channel activation." *Nature* **296**, 657 (1982)

20. White, M.M. & Benzinilla, F. "Activation of squid axon K+ channels. Ionic and gating current studies." *J. Gen Phys.* **85**, 539 (1985)

21. Sigworth, F.J. & Neher, E. "Single Na+ channel currents observed in cultured rat muscle cells." *Nature* **287**, 447 (1980)

22. Hamill, O.P., Marty, A., Neher, E., Sakmann, B. & Sigworth, F.J. "Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches." *Pflugers Arch.* **391**, 85 (1981)

23. Unwin, P.N.T. & Zampighi, G. "Structure of the junction between communicating cells." *Nature* **283**, 545 (1980)

24. Brisson, A. & Unwin, P.N.T. "Quaternary structure of the acetylcholine receptor." *Nature* **315**, 474 (1985)

25. Anderson, K., Lai, F.A., Liu, Q.V., Rousseau, E., Erickson, H.P. & Meissner, G. "Structural and functional characterization of the purified cardiac Ryanodine-receptor Ca++ release channel complex." *J. Biol. Chem.* **264**, 1329 (1989)

26. Unwin, N. "The structure of ion channels in membranes of excitable cells." *Neuron* **3**, 665 (1989)

27. Noda, M., et al., "Primary structure of *Electrophorus electricus* sodium channels deduced from cDNA sequence."*Nature* **312**, 121 (1984)

28. Tanabe, T., et al. "Primary structure of the receptor for calcium channel blockers form skeletal muscle." *Nature* **328**, 313 (1987)

29. Numa, S. "Molecular basis for the function of ionic channels." *Biochem. Soc. Symp.* **52**, 119 (1987)

30. Catterall, W.A. "Structure and function of voltage-sensitive ion channels."*Science*, **242**, 50 (1988)

31. Betz, H. "Ligand-gated ion channels in the brain: the amino acid receptor superfamily."*Neuron* **5**, 383 (1990)

32. Nicoll, R.A. "The coupling of neurotransmitter receptors to ion channels in the brain." *Science* **241**, 545 (1988)

33. Jentsch, T.J., Steinmeyer, K. & Schwarx, G. "Primary structure of *Torpedo marmorata* chloride channel isolated by expression cloning in *Xenopus* oocytes." *Nature* **348**, 510 (1990)

34. Takumi, T., Ohkubo, H. & Nakanishi, S. "Cloning of a membrane protein that induces a slow voltage-gated potassium current." *Science* **242**, 1042 (1988)

35. Kaupp, U.B. "Primary structure and functional expression from complementary DNA of the rod photoreceptor cyclic GMP-gated channel." *Nature* **342**, 762 (1990)

36. Haynes, L.W., Kay, A.R. & Yau, K.W. "Single cyclic GMP-activated channel activity in excised patches of rod outer segment membrane." *Nature* **321**, 66 (1986)

37. Zimmerman, A.L. & Baylor, D.A. "Cyclic GMP-sensitive conductance of retinal rods consists of aqueous pores." *Nature* **321**, 70 (1986)

38. Robertson, G.A., Atkinson, N.S. & Ganetzky, B. "Molecular characterization of the *slowpoke* coding region." *Neurosci. Abstr.* #281.2 (1990)

39. Greenblatt, R.E., Blatt, Y. & Montal, M. "The structure of the voltage-sensitive sodium channel." *FEBS Lett.* **193**, 125 (1985)

40. Guy, H.R. & Seetharamulu "Molecular model of the action potential sodium channel." *Proc. natn. Acad. Sci. USA* **83**, 508 (1986)

41. Noda, M., Ikeda, T., Kayano, T., Suzuki, H., Takeshima, H., Kurasaki, M., Takahashi, H. & Numa, S. "Existence of distinct sodium chanel messenger RNAs in rat brain." *Nature* **320**, 188 (1986)

42. Catterall, W.A. "Molecular properties of voltage-sensitive sodium channels." *Ann. Rev. Biochem.* **55**, 953 (1986)

43. Dekin, M.S. & Getting, P.A. "In vitro characterization of neurons in the central part of the nucleus tractus solitarius. II. Ionic basis for repetitive firing patterns." *J Neurophysiol.* **58**, 215 (1987)

44. Jan, Y.N., Jan, L.Y. & Dennis, M. "Two mutations of synaptic transmission in *Drosophila*." *Proc. R. Soc. Lond.* B **198**, 87 (1977)

45. Tanouye, M.A. & Ferrus, A. "Abnormal action potentials associated with the *Shaker* complex locus of Drosophila." *Proc. natn. Acad. Sci. USA* **78**, 6548 (1981)

46. Tanouye, M.A. & Ferrus, A. "Action potentials in normal and *Shaker* mutant Drosophila." *J. Neurogenet.* **2**, 253 (1985)

47. Salkoff, L.B. & Wyman, R. "Genetic modification of potassium channels in Drosophila *Shaker* mutants." *Nature* **293**, 228 (1981)

48. Wu, C.F. & Haugland F.N. "Voltage-clamp analysis of membrane currents in larval muscle fibers of Drosophila: alteration of potassium currents in *Shaker* mutants." *J. Neurosci.* **7**, 1307 (1985)

49. Kamb, A., Iverson, L.E., & Tanouye, M.A. "Molecular characterization of *Shaker*, a Drosophila gene that encodes a potassium channel." *Cell* **50**, 405 (1987)

50.Tempel, B., Papazian, D.M., Schwarz, T.L., Jan, Y.N. & Jan, L.Y. "Sequence of a probable potassium channel component encoded at the *Shaker* locus of Drosophila." *Science* **237**, 770 (1987)

51. Baumann A., Krah-Jentgens, H., Muller, R., Muller-Holtkamp, S., Seidel, R., Kecskemethy, N., Casal, I., Ferrus, A. & Pongs, O. "Molecular organization of the maternal effect region of the *Shaker* complex of Drosophila:

characterization of an I_A channel transcript with homology to vertebrate Na+ channel." *EMBO J.* **6**, 3419 (1987)

52.Kamb, A., Tseng-Crank, J., & Tanouye, M.A. "Multiple products at the Drosophila *Shaker* gene may contribute to potassium channel diversity." *Neuron* **1**, 421 (1988)

53. Pongs, O., Kecskemethy, N., Mueller, R., Krah-Jentzens, H., Baumann, A., Kiltz, H.H., Canal, I., Llamazares, S. & Ferrus, A. "*Shaker* encodes a family of putative potassium proteins in the nervous system of Drosophila.", *EMBO J.* **7**, 1087 (1988)

54. Schwarz, T.L., Tempel, B.L., Papazian, D.M., Jan, Y.N. & Jan, L.Y. "Multiple potassium channel components are produced by alternative splicing at the *Shaker* locus in Drosophila." *Nature* **331**, 137 (1988)

55. Pfaffinger, P., Furakawa, Y., Kubo, T., Zhao, B. & Kandel, E.R. "Molecular analysis of potassium channels in identified cells of Aplysia." *Neurosci. Abstr.* (1990)

56. Tempel, B., Jan, Y.N. & Jan L.Y. "Cloning of a probable potassium channel gene from mouse brain." *Nature* **332**, 837 (1988)

57. Baumann, A., Grupe, A., Ackermann, A. & Pongs, O. "The structure of the voltage-dependent potassium channel is highly conserved from Drosophila to vertebrate central nervous systems." *EMBO J.* **7**, 2457 (1988)

58. Ramaswami, M., Gautam, M., Kamb, A., Rudy, B. Tanouye, M.A. & Mathew, M.K."Isolation and characterization of a family of human *Shaker* potassium channels." *Mol. Cell. Neurosci.*, in press

59. Chandy, K.G., Williams, C.B., Spencer, R.H., Aguilar, B.A, Ghanshani, S., Tempel, B.L. & Gutman, G.A. "A family of three mouse potassium channel genes with intronless coding regions." *Science* **247**, 973 (1990)

60 Stuhmer, W., Ruppersburg, J.P., Schroter, K.H., Sakmann, B., Stocker, M., Giese,, K.P., Perschke, A., Baumann, A. & Pongs O. "Molecular basis of functional diversity of voltage gated potassium channels in mammalian brain." *EMBO J.* **8**, 3235 (1989)

61. Wei, A., Covarrubias, M., Butler, A., Baker, K., Pak, M. & Salkoff, L. "K+ channel diversity is produced by an extended gene family conserved in Drosophila and mouse." *Science* **248**, 599 (1990)

62. Butler, A., Wei, A., Baker, K.& Salkoff, L. "A family of putative potassium channel genes in Drosophila." *Science* **243**, 943 (1989)

63. Frech, G., Van Dongen, A.M.J., Schuster, G., Brown, A.M. & Joho, R. "A novel potassium channel with delayed rectifier properties isolated from rat brain by expression cloning." *Nature* **340**, 642 (1989)

64. McCormack T., Vega-Saenz de Miera, E. & Rudy, B. "Molecular cloning of a member of a third class of Shaker-gamily K+ channel genes in mammals." *Proc. natn. Acad. Sci. USA* **87**, 5227 (1990)

65. Vega-Saenz de Miera, E., Sen, K., Serodio, P., McCormack, T. & Rudy, B. "Description of a new class of potassium channel genes. "*Neurosci Abstr.* #6.2 (1990)

66. Timpe, B.L., Schwarz, T.L., Tempel, B.L., Papazian, D.M., Jan, Y.N. & Jan, L.Y. "Expression of functional potassium channels from *Sh* cDNA in Xenopus oocytes." *Nature* **332**, 837 (1988)

67. Iverson, L.E., Tanouye, M.A., Lester, H.A., Davidson, N. & Rudy, B. "Expression of A-type potassium channels from *Sh* cDNAs." *Proc. natn. Acad. Sci. USA* **85**, 5723 (1988)

68. Iverson, L.E. & Rudy, B. "The role of divergent amino and carboxyl domains on the inactivation properties of potassium channels from the *Shaker* gene of Drosophila." *J. Neurosci.* **10**, 2903-2916 (1990)

69. Timpe, L.C., Jan Y.N. & Jan, L.Y. "Four cDNA clones from the *Shaker* locus of Drosophila induce kinetically distinct A-type potassium currents in Xenopus oocytes." *Neuron* **1**, 659 (1988)

70. Yeh, J.Z., Oxford, G.S., Wu, C.H. & Narahashi, T. "Dynamics of Aminopyridine block of potassium channels in squid axon membrane." (1976) *J. Gen. Physiol.* **68**, 519-535

71. Meves, H. & Pichon, Y. "The effect of internal and external 4-aminopyridine on the potassium currents in intracellularly perfused squid giant axons." (1977) *J. Physiol. (London)* **268**, 511-532

72. Thompson, S. "Aminopyridine block of transient potassium current." *J. Gen Phys.* **80**, 1 (1982)

73. Kasai, H., Kameyana, M., Yamaguchi, K. & Fukuda, J. "Single transient K channels in mammalian sensory neurons." *Biophys. J.* **49**, 1243 (1986)

74. McCormack, K., Tanouye, M.A., Iverson, L.E., Lin, J.W., Ramaswami, M., McCormack, T., Campanelli, J.T. Mathew, M.K. & Rudy, B."A role for hydrophobic residues in the voltage-dependent gating of *Shaker* K+ channels." *Proc. natn. Acad. Sci. USA* , in press

75. Miller, C. "Competition for block of a Ca++-activated K+ channel by charybdotoxin and tetraethylammonium. "*Neuron* **1**, 1003 (1988)

Figure 1. Proposed Topology of Voltage-Dependent Ion Channels

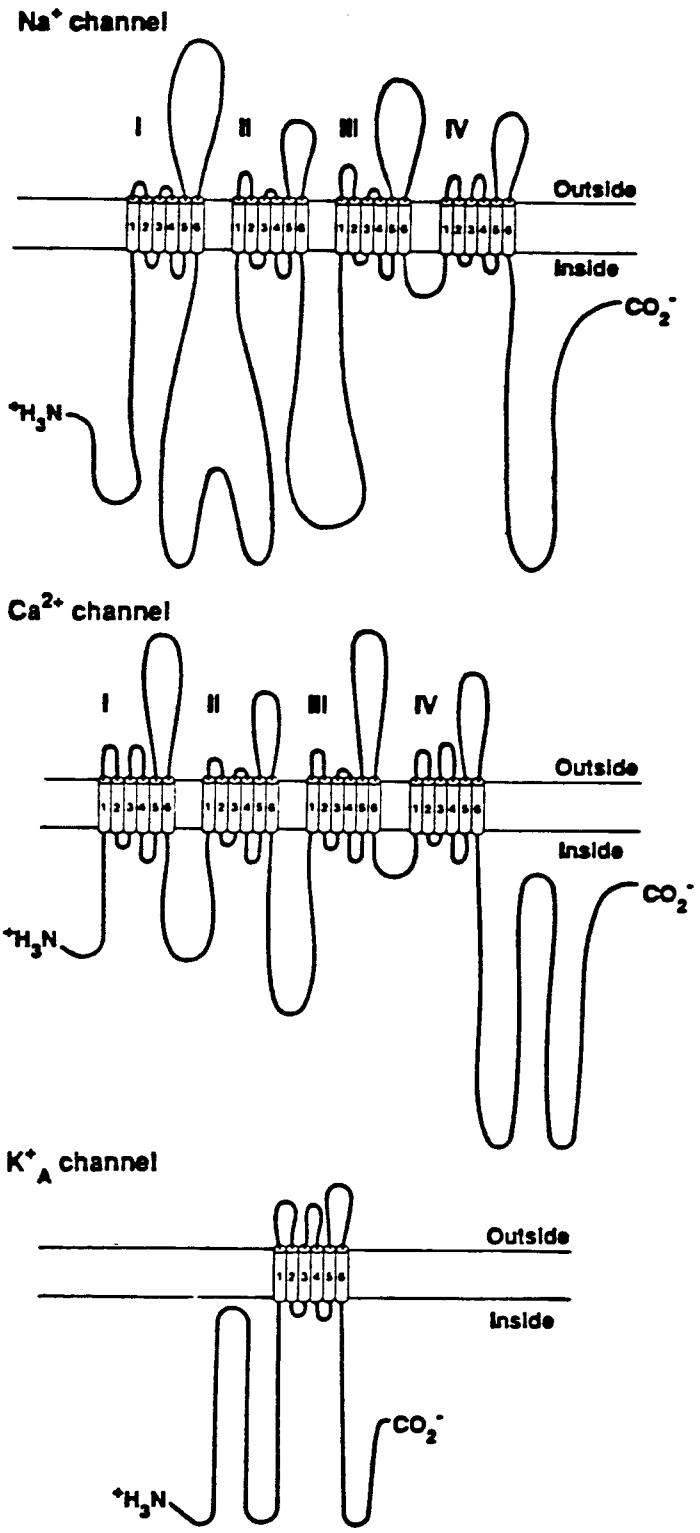


Table 1. K+ Channel Types

Voltage-dependent K channels

Delayed rectifiers [8,14,25,92,94,95]

Voltage-dependent K channel.

Main K current in many cells.

Conductance increases upon membrane depolarization, rising sigmoidally after a depolarizing step and closing exponentially upon membrane repolarization.

Inactivates slowly (hundreds of ms to s at room temp.), if at all, during a constant depolarization. Described by a first order kinetic equation raised to a power with or without a first order inactivating parameter:

$$g_K = g_K n^t k.$$

Widely distributed, with diverse kinetics, voltage-dependence and pharmacology.

Blocked by: Ca, Ba and internal TEA in most cells. 4-AP and external TEA in some cases. Ionic selectivity:

$$Tl > K > RB > NH_4 \gg Na.$$

Single channel properties (in squid giant axon,[46,120] lobster membranes[30] and skeletal muscle membranes:[182] Single channel $g \sim 15$–20 pS. Bursting behavior. Depolarization increases frequency of bursts.

Functions:
1. Action potential repolarization and thus action potential duration.
2. Contributes to refractory period.

"A" currents [2,8,43,44,92,165,194]

Fast transient voltage-dependent K currents.

Usually present in cells with delayed rectifiers and other K currents.

Conductance increases upon membrane depolarization. Inactivates fast during a step depolarization (< 100 ms at room temp). Operates around the resting potential: threshold for activation is close (usually negative to) the resting potential and steady-state inactivation is complete at the resting potential. Described by a similar equation as delayed rectifiers:

$$g_A = g_A a^t k.$$

Present in several neuronal cells, muscle and eggs with more constant kinetics, voltage-dependence and pharmacology.

Blocked by 4-AP at mM concentrations. Blocked by Cs and TEA (in).

Ionic selectivity:

$$K \gg Na$$

(unknown for other monovalent cations).

Single channel properties: Single channel $g = 15$–20 pS. Does not show voltage-dependent bursting behavior like delayed rectifier. Opening probability decreases with time, with one or two time constants. 4-AP decreases mean open time and conductance.[48,106]

Functions:
1. Latency to first spike.
2. Regulation of firing rate.
3. Action potential repolarization?

Inward rectifiers [9,80,81,84,92,150]

Also known as "Anomalous Rectifier".

Conductance increases with hyperpolarization allowing K entry. Little outflow upon depolarization. Rectification depends on (K_{out}) and it occurs around V_K.

Rectification is probably the result of block by intracellular Mg ions and not a voltage-dependent gating; possibly by Mg competing with K for entry into the channel's pore.[197]

Present in skeletal and cardiac muscle, eggs of many animals and several vertebrate and invertebrate neurons.

Usually blocked by TEA (in), Cs and Ba.

Ionic selectivity:

$$Tl > K > Rb > NH_4 \gg Na.$$

Single channel properties:
In cardiac ventricular cells is ~20 pS. Single channel conductance is ohmic in both directions of rectification.[115,197]

Functions:
1. Provides resting g in some cells, dominating outward current at Vs close to rest.
2. Maintainance of long plateaux in action potentials.
3. Cardiac pacemaker activity.
4. Egg fertilization.
5. Regulation of firing frequency.

Ca-activated K channels [29,30,90,113,115,117,153,166]

K channels activated by μM or less internal Ca.

In whole cell experiments voltage and time dependence of the K currents includes the voltage and time dependence of Ca entry into the cell.

Several types. Two main types:

I. SK channel. Small single channel conductance. Blocked by apamin.[30,166]

II. Maxi-K_{Ca} or BK channel. Very large single channel conductance. Blocked by low concentrations of external TEA and by charybdotoxin.[29,113,114,141,166]

Selectivity:

$$Tl > K > Rb > NH_4 \gg Na.$$

Both widely distributed in secretory and excitable cells.

Functions:
1. Regulate Ca entry.
2. Action potential repolarization.
3. Afterhyperpolarizations.
4. Regulation of firing rate.
5. Spike frequency adaptation.
6. Burst termination.

Neurotransmitter- and second messenger-regulated channels

"M channel"[4,5,7,8]

Slow, "non-inactivating", voltage-dependent K current. Activates close to rest or at hyperpolarized potentials.

Turned off by ACh acting on muscarinic receptors and several peptide neurotransmitters. Mediates excitation by these neurotransmitters.

Present in vertebrate central neurons and sympathetic cells.

"S channel"[99,109,179,181]

Outwardly rectifying channel with weak voltage-dependence.

Turned-off by cAMP dependent protein kinase phosphorylation resulting from [cAMP] increases produced by serotonin.

Present in *Aplysia* sensory neurons.

By closing upon serotonin action on the cell it mediates serotonin induced slow depolarization of resting potential and increase in duration of action potential which result in increase neurotransmitter release and presynaptic facilitation.

Chapter 2A

A Leucine Zipper Sequence Motif in Voltage-Gated Ion Channels

Ken McCormack*, James T. Campanelli*, Mani Ramaswami*, Mathew K. Mathew*, Mark A. Tanouye*, Linda E. Iverson$^{\Psi}$ and Bernardo Rudy$^{\zeta}$

*Division of Biology 216-76, California Institute of Technology, Pasadena, California 91125, USA

$^{\Psi}$Division of Neurosciences, City of Hope, Duarte, California 91010, USA

$^{\zeta}$Department of Physiology, New York University Medical Center, New York, New York 10016, USA

(*Nature* Vol. 340, July 13, 1989, 103)

The leucine-zipper motif, consisting of four to five leucines repeated every seventh amino-acid residue, mediates dimerization of some DNA-binding proteins[1-4] but has also been found in several other proteins, sometimes adjacent to proposed transmembrane regions[5]. Thus, as has been previously suggested for coiled coil structures[6], many protein-protein interactions may be mediated through leucine zippers, which may actually constitute a subset of the coiled coil motif[2,7]. We now report the presence of a leucine zipper in each of a set of sequences thought to represent a family of voltage-gated K+ channels and suggest its involvement in subunit interactions[8] that may mediate voltage-dependent opening and closing of the channel.

The figure shows amino-acid sequence alignment of the voltage-gated K+ channels from the region containing the leucine-zipper motif. The leucine repeat is fully conserved except in the case of Shaw but the amino-acid substitutions in this case may not prevent subunit association[1-4].

Interestingly, the leucine-zipper motif in these K+ channels is located immediately adjacent to the S4 domain. This domain is present in virtually all voltage-dependent channels and consists of the sequence Arg-X-X, repeated 4-8 times (where X is a hydrophobic amino acid and Arg can be replaced by Lys). It has been suggested that the S4 region forms an amphipathic transmembrane helix responsible for sensing membrane electrical potential[9]. In the favored model of voltage-dependent gating, changes in membrane potential result in a translocation of the S4 helix[10]. But no mechanism has been proposed by which this translocation results in opening and closing of the channel.

The intimate association of the leucine-zipper motif with the S4 domain suggests that voltage-dependent translocation of the S4 sequence alters interactions of K+ channel subunits through the leucine zipper. This alteration may take place by physically pulling the zipper into or out of the membrane and/or bending it into or out of an α-helical configuration. This would impart a voltage-dependence to subunit interactions and provide a mechanism by which S4 translocation determines open and closed channel states. We are currently testing this hypothesis using site-directed mutagenesis.

We have also found leucine zippers within the proposed M4 transmembrane segments of at least some ligand-gated channels. Na+ and Ca++ channel sequences also have leucine-zipper motifs next to their S4 domains. These motifs align with those in K+ channels but often contain prolines. It should be noted that Na+ and Ca++ channels contain a large subunit consisting of 4 homology units, each of which is equivalent to a K+ channel subunit. Voltage-dependent control of homology domain association mediated by leucine zippers may also play a role in gating of Na+ and Ca++ channels.

References

1. Kouzarides, T. & Ziff, E. *Nature* **336**, 646-651 (1988)

2. Landshultz, W.H., Johnson, P.F.& McKnight, S.L. *Science* **243**, 1681-1688 (1988)

3. Gentz, R., Rauscher, F.J., Abate, C. & Curran, T. *Science* **243**, 1695-1699 (1989)

4. Turner, R. & Tijan, R. *Science* **243**, 1689-1694 (1989)

5. Buckland, R., & Wild, F. *Nature* **338**, 547 (1989)

6. Cohen, C. & Parry, D.A.D., *Trend. Biochem. Sci.* **11**, 245-249 (1986)

7. O'Shea, E.K., Rutkowski, R. & Kim, P.S. *Science* **243**, 538-542 (1989)

8. Iverson, L.E. *et al. Proc. natn. Acad. Sci. U.S.A* **85**, 5723-5727 (1988)

9. Guy , H.R. & Seetharamulu, P. *Proc. natn. Acad. Sci. U.S.A.* **83**, 508-512 (1986)

10. Catterall, W.A. *Science* **242**, 50-61 (1988)

11. Kamb, A., Tseng-Crank, J. & Tanouye, M.A. *Neuron* **1**, 421-430 (1988)

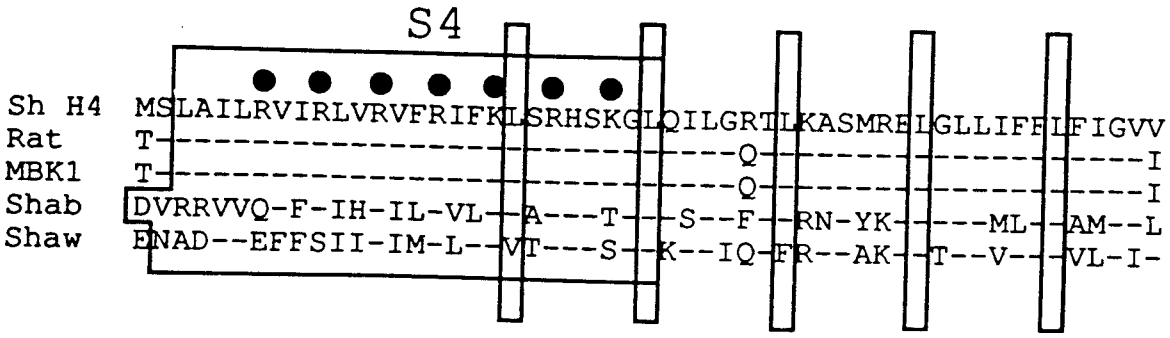
12. Baumann, A., Grupe, A., Ackermann A. & Pongs,O. *EMBO Journal* **7**, 2457-2463 (1988)

13. Tempel, B.L., Jan, Y. N. & Jan, L.Y. *Nature* **332**, 837-839 (1988)

14. Butler, A., Wei, A., Baker, K. & Salkoff L. *Science* **243**, 943-947 (1989)

Figure legend.

Amino acid alignment of K^+ channels. Shaker (Sh)[11] and rat[12] sequences have expressed functional channels in oocytes. The other sequences are from mouse (MBK1)[13], and *Drosophila* (ref. 14). Amino acids identical to Sh are depicted by dashes. Leucines of the proposed zipper are boxed. The S4 region is also boxed with circles indicating basic residues in Sh.


S4
Sh H4 MSLAILRVIRLVRVFRIFKLSRHSKGLQILGRTLKASMRELGLLIFFLFIGVV
Rat T----------Q----------I
MBK1 T----------Q----------I
Shab DVRRVVQ-F-IH-IL-VL--A---T---S--F--RN-YK-----ML--AM--L
Shaw ENAD--EFFSII-IM-L--VT---S--K--IQ-FR--AK--T--V--VL-I-

Chapter 2B

A Role for Hydrophobic Residues in the Voltage-Dependent Gating of *Shaker* K+ Channels.

Ken McCormack*, Mark A. Tanouye*, Linda E. Iverson†, Jen-Wei Lin‡, Mani Ramaswami*, Tom McCormack‡, James T. Campanelli*and Bernardo Rudy‡.

*K. McCormack, M. Ramaswami, J.T. Campanelli, M.A. Tanouye, Division of Biology 216-76, California Institute of Technology, Pasadena CA 91125

†L.E. Iverson, Division of Neurosciences, Beckman Research Institute, City of Hope, Duarte CA 91010

‡J.W. Lin, T. McCormack, B. Rudy, Department of Physiology and Biophysics and Department of Biochemistry, NYU Medical Center, 550 1st Avenue, New York, NY 10016

(*Proceedings of the National Academy of Sciences, U.S.A.*, in press)

Abstract A leucine heptad-repeat is well conserved in voltage-dependent ion channels. Here we examine the role of the repeat region in *Shaker* K+ channels through substitution of the leucines in the repeat, and through co-expression of normal and truncated products. In contrast to "leucine zipper" DNA-binding proteins, we find that the subunit assembly of *Shaker* does not depend on the leucine heptad-repeat. Instead, we report that substitutions of the leucines in the repeat produce large effects on the observed voltage-dependence of conductance-voltage and prepulse inactivation curves. Our results suggest that the leucines mediate interactions which play an important role in the transduction of charge movement into channel opening and closing.

Introduction:

The *Shaker* gene family (*Sh*) encodes proteins which produce voltage-dependent, K+-selective currents (1-3). Like other voltage-dependent ion channels, *Sh* channels open and close an aqueous pore by undergoing conformational transitions in response to changes in membrane potential. This gating behavior includes the movement of a charged component or voltage sensor (4,5). Interestingly, mutagenesis of charged residues in the S4 domain (a proposed transmembrane segment which contains 4-8 basic residues and is found in virtually all voltage-dependent ion channels including *Sh*) of the rat II Na+ channel, showed that it exhibits some of the properties expected for a voltage-sensor (6). However, the voltage-dependent gating mechanism remains unclear; in particular, it is not known how movement of the voltage-sensor(s) is transduced into the conformational transitions that result in opening and closing of the channel pore.

Functional *Sh* channels are likely to be tetramers since Na+ and Ca++ channels are composed of 4 homology domains, each roughly equivalent to a single K+ channel subunit (7). Although recent work has shown that *Sh* channels are multimeric (8) the sites of subunit interaction are unknown. *Sh* channels contain a conserved leucine-heptad repeat (5 leucines long) which overlaps two proposed transmembrane segments (S4 and S5); similar motifs are found in Na+ and Ca++ channels (9) (Fig. 1). Ion channel leucine-heptad repeats are preceded by, and partially overlap, the basic S4 domain (Fig. 1): DNA-binding "leucine zipper" proteins also contain a basic (DNA-binding) domain preceding a leucine-heptad repeat. Mutational analyses of the DNA-binding proteins have shown that leucines within the heptad repeat play a primary role in the dimerization of subunits and juxtaposition of the basic

DNA binding domains (10, 11). The functional significance of similar sequences reported in other proteins is unknown (9,12,13).

Since *Sh* K+ channels are multimeric it suggests that the leucine heptad-repeat might act as a site for subunit assembly. In addition, the close proximity of the repeat to the S4 domain suggests that the voltage-sensor may introduce a voltage dependence to the interactions mediated by residues in the leucine-heptad repeat region; gating may be a process involving the dynamics of these interactions (9). With this in mind we attempted to determine the role of the conserved leucine residues in (i) channel assembly and (ii) voltage-dependent gating.

Materials and Methods:

MUTANTS. The S4x and 102 truncations were full length 29-4 cDNAs (14, 15) with stop codons at positions Ile^{360} and Trp^{422}, respectively. E1 contains a stop codon at position Lys^{320}, and no downstream coding sequence (14). V1*n*-3 is a valine substitution at position Ile^{360}. Other substitutions are at leucines 363, 370, 377, 384 and 391, corresponding to L1-L5, respectively. All constructs were sequenced to verify the appropriate changes.

RNA SYNTHESIS AND EXPRESSION. In-vitro RNA transcripts were prepared from cDNA templates, injected into *Xenopus* oocytes and macroscopic currents were recorded using a standard two-microelectrode voltage clamp as described by Iverson et al. (1). Currents were low-pass filtered at 3 KHz. Voltage and current records were digitized and analyzed using the pCLAMP system. A 1M stock solution of 4-aminopyridine (Sigma) was made fresh and dilutions to the appropriate concentrations were

adjusted to pH 7.5 prior to use. Tetrodotoxin was kept frozen at 500uM and diluted prior to use.

Results:

Role of the Leucine Heptad-Repeat Region in Subunit Assembly. To determine if the leucine repeat region acts as a primary site for the association of subunits, we coinjected normal *Drosophila Shaker* (Sh) RNA, 29-4 (15), with several RNAs encoding truncated 29-4 proteins (Fig. 2) into *Xenopus* oocytes. A truncated, non-functional Sh product (Sh^{102}) appears to associate with normal subunits and inhibit their functional expression (16, 17). The Sh^{102} truncation occurs downstream of the leucine-heptad repeat: our truncated constructs contained stop codons at sites before (E1 or S4x) or after (102) the leucine heptad-repeat (Fig. 2). As shown, Sh products truncated before and after the zipper region inhibit 29-4 expression equally well. This suggests that the assembly of Sh subunits is not dependent on the leucine-heptad motif, in contrast to the role seen for similar sequence motifs in DNA-binding proteins. Furthermore, our results suggest that a primary site for subunit assembly lies in a region prior to the S4 segment.

Role of the Leucines in the Heptad Repeat in Voltage-Dependent Gating. Leucines in the heptad repeat (L1, L2, L3, L4 and L5) were switched individually, to valine (the V1,V2,V3,V4 and V5 mutants); alanine (A3); or both valine and alanine (V2A3). As a control, an isoleucine in S4, three residues upstream of L1, was switched to valine (V1*n*-3) (see Fig. 1). All of the mutants examined expressed well and showed similar ion selectivity (data not shown) and general kinetic behavior (see below) indicating that none of the substitutions caused gross alteration of the native channel structure.

Typical currents observed in *Xenopus* oocytes injected with 29-4 and mutant RNA are illustrated in Fig. 3. The 29-4 currents display properties similar to those reported previously (15). The V2 and V4 mutants also produce currents with transient A-type kinetics. However, V2 currents are observed only at potentials much more positive than those necessary to activate 29-4 currents while the V4 mutation has the opposite effect: V4 currents are observed at potentials more negative than those seen for 29-4. Normalized conductance-voltage relations (Fig. 3D) show that the voltages at which conductance reaches half-maximal value ($Vm^{1/2}$), is shifted about +70 mV for V2 and -25 mV for V4. The V1 mutation produces a depolarizing shift like that of V2 but of larger magnitude, while the conductance-voltage curves of V3 and V5 are shifted in the same direction as V4 (Table 1). Shifts in voltage dependence for substitutions of L3 appear to be due to the degree of hydrophobicity, or the chain length, rather than steric constraints since an alanine substitution (A3) causes a larger voltage shift than V3. The observed shifts in the conductance-voltage curves suggest that the leucines play an important role in determining the relative stabilities of open and closed states of the channel. In addition, all of the leucine substitutions have parallel shifts in the prepulse inactivation curves (Fig. 3E, Table 1), supporting the idea that inactivation is coupled to channel opening (18).

In order to test the possibility of alterations in the closed states of mutant channels we examined the sensitivity of two of the mutants to the state-dependent K+ channel blocker, 4-aminopyridine (4-AP), which has been suggested to bind preferentially to channels in the closed state (19, 20). V2 currents are more sensitive while V4 currents are more than two orders of magnitude less sensitive than the wild type currents (Fig. 2F). Although it is possible that these differences may be due to modifications in the binding site

for 4-AP, they are consistent with increased and reduced lifetimes for closed states in the V2 and V4 mutants, respectively. In particular, the large decrease in 4-AP sensitivity in V4 channels may indicate that a closed state(s) which preferentially binds 4-AP is very short-lived in these channels.

Both the V1 and V2 currents show dramatic decreases in the slopes of the conductance-voltage and prepulse inactivation curves. Mutations of the other heptad-leucines (V3, A3, V4 and V5) cause little apparent change in slope (Fig. 3 , Table 1). Since the slope of the steady-state inactivation curve has been suggested to be a better indicator for the number of charges moved during the activation process in Sh currents (18), the apparent number of "gating charges" is calculated from this slope in Table 1. The total apparent gating charge of a 29-4 channel is approximately 7 while it is reduced approximately 3-fold for V2 and 5-fold for V1.

As in previous reports of Sh currents (15), 29-4 and the mutant currents exhibit a rapidly-inactivating and a non-inactivating component. Although the substitutions have little effect on the rate of the rapidly-inactivating component (Table 1) V1 and V2 currents alter the relative magnitude of the non-inactivating component. These changes are not due to a shift in voltage-dependence, since they are also present at high membrane potentials (see Fig. 3B). Single channel analysis of V2 (Fig. 4) indicates that the differences in macroscopic inactivation cannot be explained by an increase in mean open duration (0.81 ± .21 ms, n=6, independent of membrane potential, for V2; compared to 1.05 ms for 29-4 (ref. 15)): the observed decrease in mean open duration suggests that the stability of the open-channel pore in V2 is decreased. The experiment also shows that the mean number of bursts per depolarizing pulse in V2 (2.28 at +20 mV, 1.8 at +40 mV and 1.92 at +100 mV) is not significantly affected by voltage. This is similar to wild type 29-4

currents (15). However, for V2 there is an increase in the mean number of bursts per depolarizing pulse with openings; the mean value for V2 is 2.05 ± .21 (n = 7) while the value for wild-type 29-4 is 1.37 ± .14 (n = 5). Thus, an increased probability of channel reopening appears to account for the increase in the non-inactivating component, perhaps, by decreasing the stability of an inactivated state. One possibility is that amino acid residues in the heptad-repeat region are closely associated with the receptor for a blocking particle as proposed in some models of channel inactivation (5, 21). This receptor is thought to reside on or near the internal mouth of the pore. The alterations in both the probability of channel reopening and mean open duration suggest that the conserved leucine-heptad repeat region (thought to be located on the internal side of the membrane) may itself form a portion of the channel pore.

The effects of the mutations appear to define two functional classes for the heptad-leucines: class I mutations (V1, V2) appear to impair transitions from closed to open states, thereby causing shifts in $Vm^{1/2}$ and $Vh^{1/2}$ to more positive potentials, decrease the slopes of conductance-voltage and prepulse inactivation curves, and the extent of inactivation. Class II mutations (V3, A3, V4, V5) appear to facilitate transitions from closed to open states, thereby causing shifts in $Vm^{1/2}$ and $Vh^{1/2}$ to more negative potentials with lesser effects on slope and the extent of inactivation. To determine how mutations in the different classes interact, we constructed the double mutant V2A3. The double mutant has $Vm^{1/2}$, $Vh^{1/2}$ and Iss values which are intermediate between those for the V2 and A3 single mutants (Table 1). In contrast, the slopes of the conductance-voltage (data not shown) and prepulse inactivation curves (Table 1) for the double mutant resemble that of V2, indicating that this effect is dominant, and that the alterations in slope do not result

indirectly from the voltages at which the channel opens or inactivates, or from changes in Iss.

Discussion:

Hydrophobic Amino Acids Are Important in Stabilizing Open-Closed Conformations. While leucine substitutions have little effect on other properties, some of the alterations in the conductance-voltage and prepulse inactivation curves are quite large. Our results show that conservative substitutions of hydrophobic amino acids in the S4 domain produce voltage shifts similar to, but of greater magnitude than, substitutions of the positively charged residues (6, 22, 23, 24). For example, the 100mV shift in $Vm^{1/2}$ and approximate five-fold decrease in slope for the V1 mutant are striking. Furthermore, since these alterations occur without any net change in charge, the large decreases in slope (in V1 and V2) are particularly interesting. These slope changes may reflect large reductions in gating charge resulting from alterations in either the positioning of S4 charges (or dipole) relative to the electric field vector or in the ability of subunits to reach their fully-activated conformation. However, given the magnitude of slope differences it is difficult to explain in either of these terms how V1 and V2 channels could achieve open channel conformations at all. One alternative possibility is that these slope changes may not reflect changes in charge movement of the voltage-sensor. Furthermore, gating may require cooperative or other allosteric transitions; if residues in the leucine-heptad repeat region, particularly L1 and L2, are critical in mediating the interactions underlying these allosteric transitions, then even conservative substitutions (V1 and V2) could alter activation and the apparent gating charge.

Our data are consistent with the idea that the S4 domain (V1 and V2) is important in determining the observed voltage-dependence of channel opening, however, mutations of leucines in the next membrane spanning domain (V4 and V5), S5, and in the linker region between S4 and S5 (A3, V3), also produce voltage shifts. In contrast, substitution of a valine for an isoleucine residue in S4, three residues upstream of L1 (V1n-3), and outside the defined leucine-heptad repeat (see Fig. 1), results in no apparent changes in any of these properties (Table 1). Thus, not all hydrophobic amino acids in or near the S4 domain play a similar role to those in the leucine heptad repeat. Taken together, these data suggest that interactions mediated by heptad-leucines are important in stabilizing conformational states of the channel and that these interactions may strongly influence the transduction of charge movement into channel opening and closing. Leucines in the heptad repeats of Na+ and Ca++ channels may play similar roles. For example, in the rat IIa Na+ channel, substitution of L1 (in the second homology domain) by a phenylalanine results in a voltage shift of +25 mV while substitution of six other residues, including charged or transmembrane residues, had no effect (22).

Significance of the Heptad Repeat. The asymmetry of effects in the two different classes of mutations may indicate that the first two and the last two or three leucines in the motif stabilize different conformational states of the channel. This is consistent with the idea that the leucines are located in two separate (possibly transmembrane) domains. Given the fact that the V1 and V2 mutations have similar effects, and the frequent occurrence of helical transmembrane segments, our results suggest that L1 and L2 may be aligned along one face of a helix to interact with other channel structures which stabilize conformational states of S4. The similar effects of the V4 and V5

mutants also suggest the possibility of helical structure in this portion of the S5 domain. In the Sh^5 mutation, substitution of a phenylalanine, located between L4 and L5, by isoleucine produces a voltage-shift in the opposite direction of V4 and V5 (17, 27): this residue would be located on the opposite face of an alpha-helix from that of L4 and L5. It will be interesting to correlate the mutagenic effects of other residues in and around the heptad-repeat region as a function of the helical face on which they may lie.

Two issues which remain unclear are: (i) why L3, and the seven residue periodicity in the cytoplasmic linker between the S4 and S5 segments are highly conserved in voltage-gated ion channels, and (ii) why conservative hydrophobic substitutions in a putative transmembrane domain (V1 and V2) have such large effects on the conductance-voltage and prepulse inactivation curves. In the DNA-binding leucine zipper proteins, dimerization mediated by the heptad-repeat region appears to be a contiguous coiled coil association of two alpha-helices (28); dimerization takes place largely through the buried leucine (hydrophobic) residues along one face of the respective helices.

Helical projections of the heptad repeat region of *Drosophila Shaker* (Sh), *Sh* family members, and Na+ channels (Fig. 5) demonstrate that the arms of one face of the helix, including the leucine arm, are conserved while the arms of the opposite face are not. Thus, this region may be involved in forming coiled coil associations. Although a contiguous helix containing the five leucines of the repeat is inconsistent with the positioning of both the S4 and S5 domains perpendicular to the membrane, it is striking that a specific type of structural organization, with an approximate 3.5 residue periodicity, has been conserved in this region in many species and many types of voltage-dependent ion channels. One possibility is that the portion of the S4 domain which contains L1 and L2 may not be located in the membrane in all channel

conformational states; residues located between the charged residues in this portion of S4 are less hydrophobic than those in the remaining portion of S4 supporting this possibility. Thus, the heptad-repeat region may form contiguous helices in some conformational states. In addition, the large voltage shifts and slope alterations observed in V1 and V2 also suggest that this portion of the S4 domain may be in an aqueous environment since the energetics of leucine-mediated hydrophobic interactions might be relatively large in such an environment in comparison to the membrane interior. It is also possible that they lie in or near the aqueous environment of the channel pore.

Substitution of heptad leucines resulted in alterations in the "effective" voltage-dependence of channel opening. This is consistent with the idea that the voltage-dependent conformational changes of gating-charge movement may be transduced through subunit interactions mediated by the leucine-heptad repeat region into opening of the channel pore (9). In addition, alterations in inactivation and in the stability of the channel pore were observed suggesting that the conserved heptad-repeat region, itself, may be involved in determining a portion of the channel pore.

We thank H. A. Lester, N. Davidson, X.C. Yang and R. Dunn for kindly providing rat IIa Na channel RNA; and H.A.L. for oocytes; D. Rees and T. Kouzarides for helpful discussions; W.N. Zagotta and R. Aldrich for providing a manuscript prior to publication; and R. McMahon for expert technical assistance. This research was supported by U. S. Public Health Service Grants NS21327 and GM42824 to M.A.T.; GM26976 to B.R.; NS28135 to LI and GM29836 to H.A. Lester.

1. Iverson, L.E., Tanouye, M.A., Lester, H.A., Davidson, N. & Rudy, B. (1988) *Proc. Natn. Acad. Sci. U.S.A.* **85**, 5723-5727

2. Stuhmer, W., Ruppersburg, J.P., Schroter, K. H., Sakman, B., Stocker, M., Giese, K. P., Perschke, A. & Pongs, O. (1989) *EMBO Journal* **8**, 3235-3244

3. Wei, A., Covarrubias, M., Butler, A., Baker, K., Pak, M. & Salkoff, L. (1990) *Science* **248**, 599-603

4. Hodgkin, A.L. & Huxley, A.F. (1952) *J. Physiol. (Lond.)* **117**, 500-544

5. Armstrong, C.M. (1981) *Physiol.Rev.* **61**, 644-683

6. Stuhmer, W., Conti, F., Suzuki, H., Wang, X., Noda, M., Yahagi, N., Kubo, H. & Numa, S. (1989) *Nature (London)* **339**, 597-603

7. Numa, S. (1986) *Biochem. Soc. Symp.* **52**, 119-143

8. McCormack, K., Lin, J. W., Iverson, L. E. & Rudy, B. (1990) *Biochem. Biophys. Res. Comm* **171**, 1361-1371

9. McCormack, K., Campanelli, J. T., Ramaswami, M., Mathew, M. K., Tanouye, M. A., Iverson, L. E., & Rudy, B. (1989) *Nature (London)* **340**, 103

10. Kouzarides, T. & Ziff, E. (1988) *Nature (London)* **336**, 646-651

11. Scheurmann, M., Neuberg, M., Hunter, J.B., Jenuwein, T., Ryseck, R.P., Bravo, R. & Muller, R. (1989) *Cell* **56**, 507-516

12. Buckland, R. & Wild, F. (1989) *Nature (London)* **338**, 547

13. White, M.K. & Weber, M.J. (1989) *Nature (London)* **340**, 103

14. Kamb, A., Tseng-Crank, J. & Tanouye, M.A. (1988) *Neuron* **1**, 421-430

15. Iverson, L.E. & Rudy, B. (1990)*J. Neuroci.* **10**, 2903-2916

16. Gisselman, G., Sewing, S., Madsen, B. W., Mallart, A., Angrat-Petit, D., Muller-Holtkamp, F., Ferrus, A. & Pongs, O. (1989) *EMBO Journal* **8**, 2359-2364

17. Gautam, M., & Tanouye, M. A. (1990) *Neuron* **5**, 67-73

18. Zagotta, W.N & Aldrich, R.W. (1990) *J. Gen. Physiol.* **95**, 29-60

19. Yeh, J.Z., Oxford, G.S., Wu, C.H. & Narahashi, T. (1976) *J. Gen. Physiol.* **68**, 519-535

20. Meves, H. & Pichon, Y. (1977) *J. Physiol. (Lond.)* **268**, 511-532

21. Hoshi, T., Zagotta, W. N. & Aldrich, R. W. (1990) *Science* **250**, 533-538

22. Auld, V.J., Goldin, A.L., Krafte, D.S., Catterall, W., Lester, H. A., Davidson, N. & Dunn, R. (1990) *Proc. Natn. Acad. Sci. U.S.A.* **87**, 323-327

23. Papazian, D. M., Timpe, L.C., Jan, Y.N. & L.Y. Jan, (1989)*Soc. Neurosci. Abstr.* **15**, 337

24. McCormack, K., Ramaswami, M., Mathew, M. K., Tanouye, M. A., Iverson, L. E., McCormack, T. & Rudy, B. (1989) *Soc. Neurosci. Abstr.* **15**, 337

25. Zagotta, W.N & Aldrich, R.W. (1990) *J. Neurosci.* **10,** 1799-1810

26. O'Shea, E.K., Rutkowski, R. & Kim, P.S. (1989)*Science* **243**, 538-542

27. Ramaswami, M., Gautam, M., Kamb, A., Rudy, B., Tanouye, M. A. & Mathew, M.K. *Cell. Mol. Neurosci.* in press

28. Tanabe, T., Takeshima, H., Mikami, A., Flockerzi, V., Takahashi, H., Kangawa, K., Kojima, M., Matsuo, H., Hirose, T. & Numa, S. (1987) *Nature (London)* **328**, 313-318

Figure 1. Deduced amino acid alignments of the leucine-heptad repeat region. Basic residues in S4 (stars) and leucine residues in the heptad repeat (boxed) were aligned. Amino acids identical to Sh are indicated by dashes. Proposed transmembrane segments S4 and S5 are indicated. Sh (14), is the *Drosophila Shaker* channel. Other K+ channel sequences are a rat Sh homolog (RCK1) (2), two human Sh homologs (HukI and HukII) (32) and *Shab*, *Shaw* and *Shal* which represent other *Drosophila* K+ channel genes (3). Na and Ca sequences are from the second homology domains of the rat IIa Na+ channel (25) and the skeletal muscle dihydropyridine receptor (33), respectively.

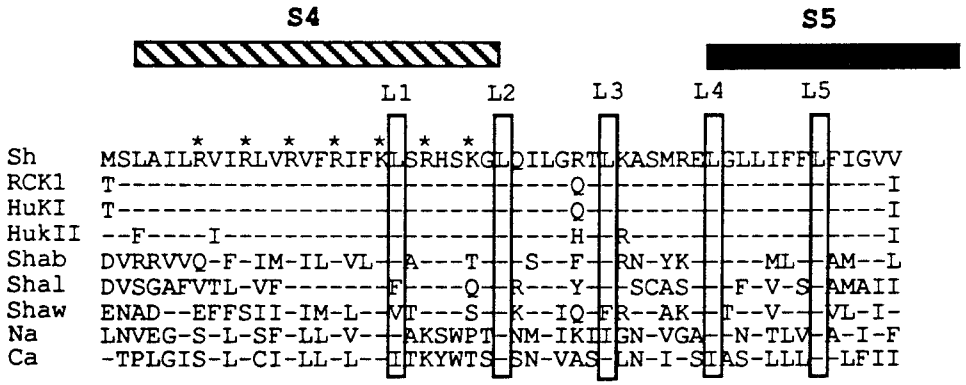
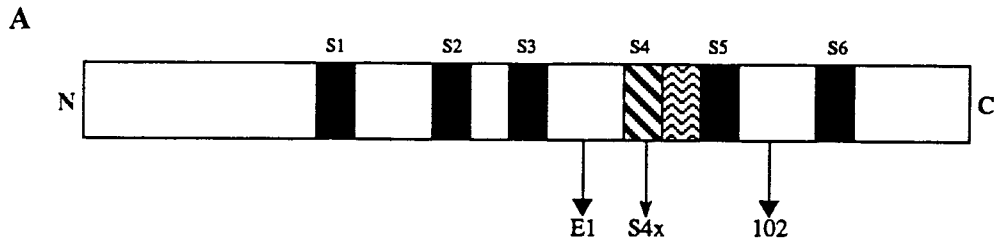
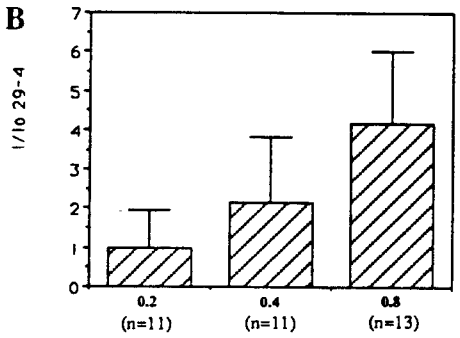
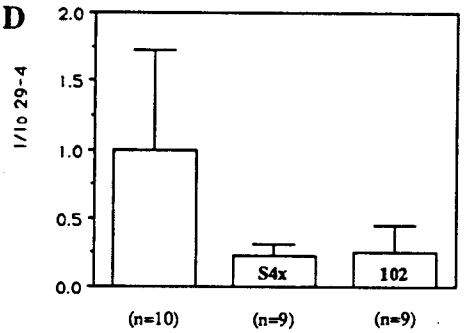

S4
S5
L1
L2
L3
L4
L5
Sh MSLAILRVIRLVRVFRIFKLSRHSKGLQILGRTLKASMRELGLLIFFLFIGVV
RCK1 T----------Q----------I
HuKI T----------Q----------I
HukII --F----I----------H--R----------I
Shab DVRRVVQ-F-IM-IL-VL--A---T---S--F--RN-YK-----ML--AM--L
Shal DVSGAFVTL-VF-------F----Q--R---Y---SCAS---F-V-S-AMAII
Shaw ENAD--EFFSII-IM-L--VT---S--K--IQ-FR--AK---T--V----VL-I-
Na LNVEG-S-L-SF-LL-V---AKSWPT-NM-IKIIGN-VGA---N-TLV-A-I-F
Ca -TPLGIS-L-CI-LL-L--ITKYWTS-SN-VAS-LN-I-SIAS-LLL---LFII

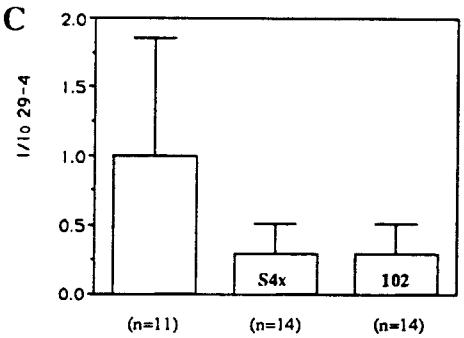
Figure 2. A) Schematic diagram of a Sh subunit. S1 - S6 denote potential membrane-spanning segments; the leucine heptad repeat overlaps S4 and S5. The locations of E1, S4x and 102 C-terminal truncations are indicated. B) Normalized currents of 29-4 at the indicated concentrations of RNA (ng/oocyte). Expression of currents is linear over the concentration ranges examined; the ability of the oocytes to translate and assemble Sh 29-4 products is apparently not limiting at these concentrations. C) Oocytes were injected with 29-4 RNA (0.2 ng) alone or coinjected with 29-4 (0.2 ng) and the indicated truncated (0.4 ng) RNA. The truncated RNAs eliminated about 70% of the expressed 29-4 current. The S4x and 102 truncations inhibited 29-4 currents equally well; similar results were seen with coinjections of E1 (data not shown). No alterations were seen in voltage-dependent or kinetic behavior for the currents expressed in coinjected oocytes. In panels D and E we demonstrate that the inhibitory effects of the truncations are specific to the 29-4 currents; Na+ channel expression is unaffected. D) Coinjections of 29-4 (0.25 ng) and rat IIa Na+ channel (1.0ng) RNAs; or 29-4 (0.25 ng), rat IIa Na+ channel (1.0 ng) and the indicated truncated (0.50 ng) RNA. Recordings were made in the presence of tetrodotoxin (500 nM) to block Na+ currents. Truncated RNA inhibited the tetrodotoxin-insensitive current by about 75%. E) Similar experiment to D) but the oocytes were recorded from in the presence of 4-aminopyridine (4mm) to block Sh currents. These data suggest that the truncated products specifically associate with Sh subunits. All currents were leak subtracted (15) and normalized to currents obtained when no truncations were injected. All experiments were done on the same batch of oocytes 3 days after injection and within an 8 hour period. Oocytes were

held at -90 and stepped to +70 mV for 29-4 and -10 mV for rat IIa (M860) (ref. 25) currents. All data are mean (+s.d.).


A
S1
S2
S3
S4
S5
S6
N
C
E1
S4x
102


B
I/Io 29-4
0.2
(n=11)
0.4
(n=11)
0.8
(n=13)


D
I/Io 29-4
S4x
102
(n=10)
(n=9)
(n=9)


C
I/Io 29-4
S4x
102
(n=11)
(n=14)
(n=14)

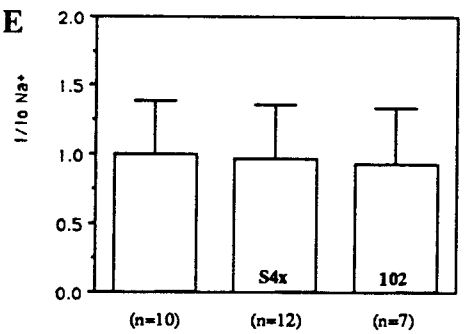

E
I/Io Na+
S4x
102
(n=10)
(n=12)
(n=7)

Figure 3. Macroscopic currents from a *Xenopus* oocyte injected with 29-4 (A), V2 (B) or V4 (C), RNA. Oocytes were held at -90 mV stepped to a -120 mV prepulse for 1 s and then depolarized from -60 to 100 mV in 20 mV increments. The time between episodes was 10 s. Only the currents during the depolarizing pulses are shown. For parts D-H the following symbols are used for 29-4 (open squares), V4 (open triangles), V2 (open circles) and V1 (filled circles) currents. D) A similar protocol (above) but in 10mV increments was used to plot conductance-voltage relations. Conductance was determined by g=Ip/(V-Vk) where Ip is the peak current at voltage V and Vk, the K+ equilibrium potential, is taken as -90mV. Conductances were normalized (g/gmax) by dividing the conductance at the indicated potential by the maximum observed conductance. Currents (Ip) were obtained after leak subtraction (15). Data are mean ± s.d., where n = 15, 7 and 5 for 29-4, V2 and V4, respectively. E) Normalized prepulse inactivation. Oocytes were held at -100 mV, stepped for 500 ms to the indicated prepulse voltages and then depolarized to a test pulse of 30 (29-4 and V4) or 80 mV (V2). The time between episodes was 10 s. Peak currents obtained during the test pulse following a prepulse to the indicated voltage were divided by the peak currents obtained during a test pulse following a prepulse to -80 mV. Data are mean ± s.d. where n = 9, 4 and 4 for 29-4, V2 and V4, respectively. F) 4-aminopyridine (4-AP) sensitivities of 29-4, V2 and V4. 4-AP was continuously circulated through the recording chamber. The effect of 4-AP was monitored with a series of voltage steps (1/min.) until no further change was observed (usually 5-10 min.).The peak currents at 20 mV (29-4 and V4) or 70 mV (V2) at the indicated concentrations of 4-AP were divided by the peak currents at the same voltage in the absence of 4-AP. Data are mean ± s.d. where n = 4, 4,

and 5 for 29-4, V2 and V4, respectively. G) The log of the normalized conductance (cf. Fig. 3D) of the indicated Sh constructs. To facilitate comparisons of slope, relative voltages are used, with $Vm^{1/2}$ for each construct taken as 0 mV. All values are means where n = 15, 5, 7 and 5 for 29-4, V1, V2 and V4 respectively. H) Steady-state inactivation relationships for the indicated constructs (cf. fig. 2E). To facilitate comparisons of slope $Vh^{1/2}$ is defined as 0 mV for each of the constructs. All values are means where n = 9, 4, 4 and 4 for 29-4, V1, V2 and V4, respectively.

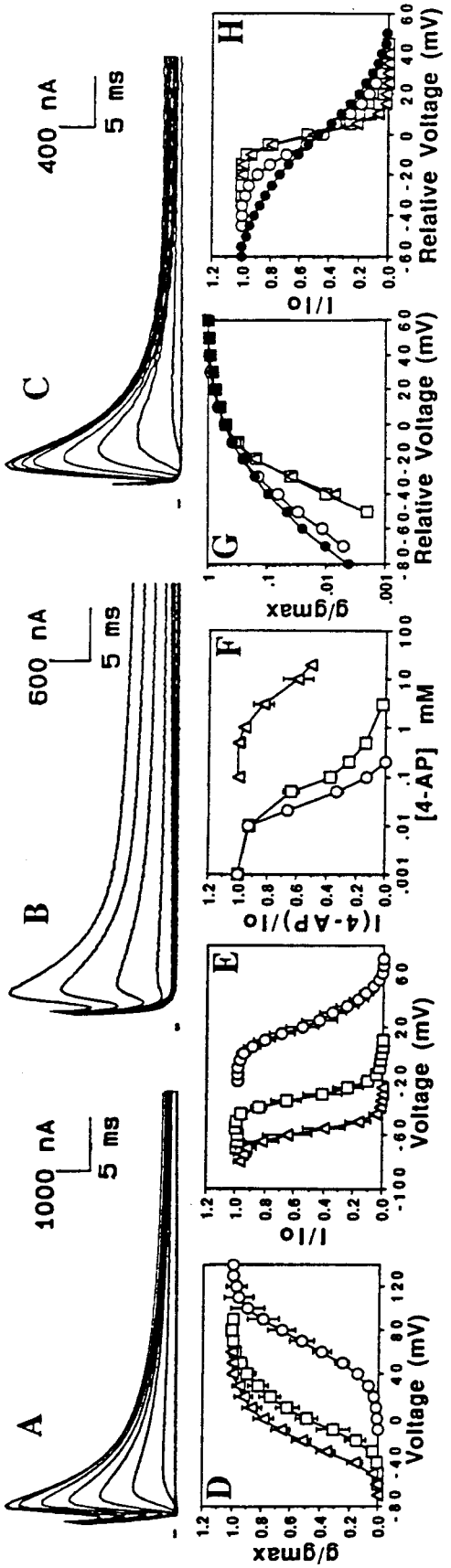

A
1000 nA
5 ms
B
600 nA
5 ms
C
400 nA
5 ms
D
g/gmax
Voltage (mV)
E
I/Io
Voltage (mV)
F
I(4-AP)/Io
[4-AP] mM
G
g/gmax
Relative Voltage (mV)
H
I/Io
Relative Voltage (mV)

Figure 4. Single channels in cell-attached patches (15) from an oocyte injected with V2 RNA. Shown are nine representative sweeps at depolarizations to +20 mV (A), +40 mV (B), or +100 mV (C), from a holding potential of -100 mV. The depolarizing pulse (40 ms) is shown above each set of records. The minimum closed time used to distinguish bursts from individual openings was 2 ms. Each experiment consisted of 64 sweeps. The average of sweeps with no openings was used to subtract leak and uncompensated capacitative currents. Voltage steps were delivered every 5s. The currents were low-pass filtered through an 8-pole Bessel filter at 2 KHz. Data were digitized at 50us per point.

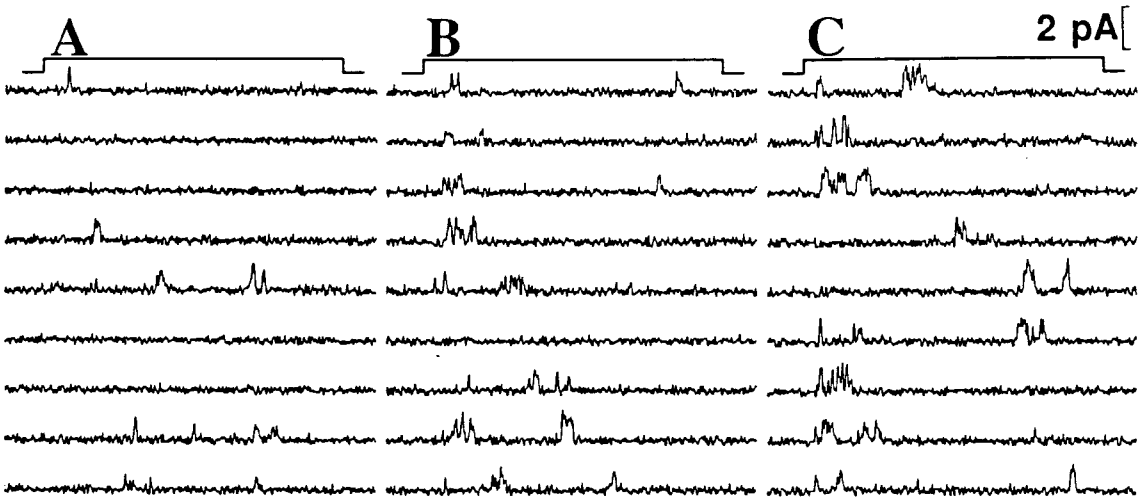
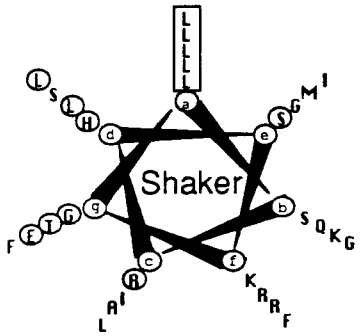
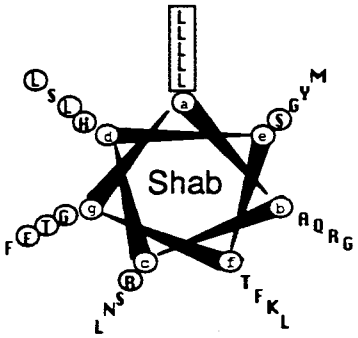
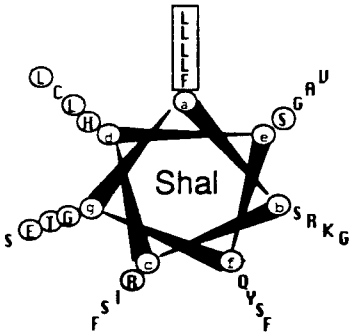
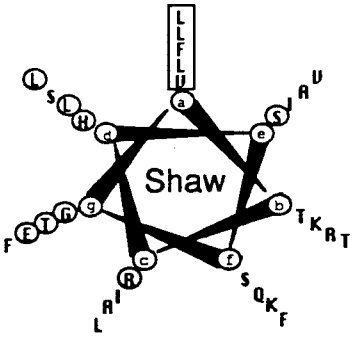

A
B
C
2 pA

Figure 5. Helical projections of the indicated sequences (aligned in Fig. 1). Residues of the heptad repeat are boxed and residues that are conserved among all of the K+ channel sequences and the indicated sequence are circled. Arms a, d and g are well conserved among all published K+ channel sequences. Adjacent arms a and d which could potentially mediate coiled-coil interactions are well conserved even in Na+ channel sequences.


Shaker


Shab


Shal


Shaw

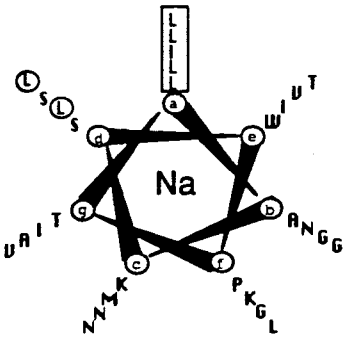

Na

Table 1. Physiological properties of substitution mutants. $Vm^{1/2}$ is the voltage at half-maximal conductance (cf. Fig. 3D). The ratio of peak current (Ipeak) and steady-state current (Iss, the current values remaining at the end of a 50 ms test pulse) was determined for test potentials standardized for each mutant at 30 mV above $Vm^{1/2}$ (cf. Fig. 3A). Time constants of inactivation (T1 and T2) were obtained by fitting the decay of currents to a steady-state value with the double exponential function: $I_t/I_o = [A1\ exp^{-t/T1} + A2\ exp^{-t/T2}]$ where I_t is the current at time t, I_o is the extrapolated value of the peak current at t=0, and A1 + A2 = 1. The data were obtained from currents during a 50 ms test pulse to 40 mV above Vm1/2 following a 1s prepulse to -120 mV. Steady-state inactivation curves were fitted to a Boltzman distribution:

$$I/Imax = 1/[1 + e^{(V-Vh1/2)Vhslope^{-1}}]$$

where $Vh^{1/2}$ is the midpoint of inactivation and the Vh slope factor is expressed as the number of mV to produce an e-fold change in I/Imax. Prepulse inactivation curves were obtained as in Fig.3 using test potentials of 30mV except for V1 (100 mV), V2 (80 mV) and V2A3 (60mV). Charge # is the number of apparent gating charges as calculated from: charge # = RTF^{-1}(Vh slope)$^{-1}$. The number of oocytes from which the data in the preceding columns was obtained is n. Data shown are means (± s.d.) for the *Sh* construct indicated.

Table 1

Mutant	Vm 1/2 (mV)	Iss/Ipeak	T1 (ms)	T2 (ms)	A1/A1+A2	n	Vh 1/2 (mV)	Vh slope (mV/e fold)	charge #	n
wild type	1 ± 5	.14 ± .05	6.0 ± 1.1	26 ± 11	.75 ± .03	15	-34 ± 1	3.7 ± 0.5	6.8	9
V1	100 ± 6	.46 ± .07	5.3 ± 1.8	172 ± 185	.65 ± .26	5	48 ± 4	17.4 ± 1.9	1.5	4
V2	69 ± 7	.26 ± .01	5.4 ± 1.2	51 ± 22	.84 ± .08	7	26 ± 3	11.0 ± 0.9	2.3	4
V3	- 5 ± 2	.11 ± .02	5.3 ± 0.2	---	1	3	-41 ± 2	3.2 ± 0.1	7.9	3
V4	- 22 ± 3	.13 ± .03	8.4 ± 1.5	---	1	5	-58 ± 6	3.6 ± 0.5	7.0	4
V5	- 7 ± 7	.12 ± .05	6.9 ± 1.1	---	1	5	-45 ± 4	4.0 ± 0.2	6.3	5
A3	-19 ± 3	.08 ± .02	5.2 ± 0.6	---	1	5	-50 ± 4	3.1 ± 0.5	8.1	5
V2A3	32 ± 5	.16 ± .02	9.1 ± 1.9	---	1	6	-9 ± 4	11.1 ± 3.0	2.3	6
V1n-3	3 ± 5	.14 ± .05	7.0 ± 1.1	31 ± 9.3	.76 ± .04	4	-32 ± 6	3.6 ± 0.3	7.0	3

Chapter 3

Shaker K^+ Channel Subunits Form Heteromultimeric Channels With Novel Functional Properties

Ken McCormack[1], Jen-Wei Lin[2], Linda E. Iverson[3] and Bernardo Rudy[2]

[1]Division of Biology 216-76, California Institute of Technology, Pasadena, CA, 91125

[2]Department of Physiology, New York University Medical Center, New York, NY 10016

[3]Division of Neurosciences, City of Hope, Duarte, CA 91010

(*Biochemical and Biophysical Research Communications* Vol. 171, No. 3, 1361-1371, September 28, 1990)

Abstract

A large number of related genes (the *Sh* gene family) encode potassium channel subunits which form voltage-dependent K^+ channels by aggregating into homomultimers. One of these genes, the *Shaker* gene in *Drosophila*, generates several products by alternative splicing. These products encode proteins with a constant central region flanked by variable amino and carboxyl domains. Co-injection of two *Shaker* RNAs with different amino or different carboxyl ends into *Xenopus* oocytes produces K^+ currents that display functional properties distinct from those observed when each RNA is injected separately, indicating the formation of heteromultimeric channels. The analysis of *Shaker* heteromultimers suggests certain rules regarding the roles of variable amino and carboxyl domains in determining kinetic properties of heteromultimeric channels. Heteromultimers with different amino ends produce currents in which the amino end that produces more inactivation dominates the kinetics. In contrast, heteromultimers with different carboxyl ends recover from inactivation at a rate closer to that observed in homomultimers of the subunit which results in faster recovery. While this and other recent reports demonstrate that closely related *Sh* family proteins form functional heteromultimers, we show here that two less closely related *Sh* proteins do not seem to form functional heteromultimeric channels. The data suggest that sites for subunit recognition may be found in sequences within a core region, starting about 130 residues before the first membrane spanning domain of *Shaker* and ending after the last membrane spanning domain, which are not conserved between Sh Class I and Class III genes.

Introduction

K^+ channels comprise an extremely diverse family of ion channels (1-6). The choice of which channel types are expressed in a cell helps determine the cell's membrane potential and its modulation by the microenvironment. In excitable cells, in addition, the selection of which K^+ channels are expressed helps to determine action potential waveforms and firing patterns.

Recently, more than ten distinct but related genes (the *Sh* gene family) encoding voltage-dependent K^+ channels have been identified (7-24). These genes encode K^+ channel subunits which produce voltage-dependent K^+ channels in *Xenopus* oocytes and in cell lines (see also: 25-29). The channels expressed by these genes are probably homomultimers, perhaps tetramers, of the expressed subunits (7, 25-27, 30). If K^+ channels are formed by aggregation of subunits, an important question is whether distinct K^+ channel subunits can interact to form heteromultimeric channels with novel functional properties. Heteromultimer formation may be another molecular mechanism to generate functional diversity of K^+ channels.

In the case of the *Shaker* gene in *Drosophila*, the first cloned K^+ channel gene, several products are generated by alternative splicing (9-11). The proteins predicted by the alternatively spliced transcripts consist of a central constant region flanked by variable amino and carboxyl termini. When injected individually into *Xenopus* oocytes these transcripts produce channels with similar properties of voltage-dependence, K^+ selectivity and pharmacology (25-28, 35) but differ in their kinetic properties; particularly the kinetics of onset and recovery from inactivation. The first question we address in this paper is whether heteromultimeric channels, with novel kinetic properties, are formed when transcripts encoding subunits with

different amino or different carboxyl termini, are co-injected into *Xenopus* oocytes.

The various genes in the *Sh* family can be divided into several groups or classes based on sequence homology and, hence, evolutionary relatedness. Members of a given class are much more similar to each other, even among different species, than members of distinct classes within the same species (14, 16, 19, 22-24). K^+ channel subunits of distinct classes show amino acid sequence differences in regions of the protein which are very conserved among subunits of the same class. The second question we address here is whether heteromultimer formation can take place between two K^+ channel subunits of different classes. This and other recent reports (31-34) provide evidence of heteromultimer formation among distinct pairs of *Sh* family gene products of the same class. Heteromultimer formation confirms the idea that these proteins are subunits of multimeric K^+ channels. We also show that subunits from two distinct classes appear not to form heteromultimers. These results identify probable regions of subunit recognition and provide clues as to how the various subunits expressed in a cell may lead to the complement of functional channels present in that cell.

Experimental Procedures

In-vitro synthesis of RNA. Recombinant Bluescript (Stratagene, San Diego, CA) plasmids containing the cDNA inserts are described elsewhere (23, 35, 36). Plasmids were linearized, and full-length capped RNA transcripts synthesized using T7 or T3 RNA polymerase (23, 35). The in-vitro transcribed RNA was stored in H_2O at a concentration of 20-200 ng/ul at -70°C. RNA mixtures were

made just prior to injection. A 1:1 mixture means that equal amount of RNA were mixed.

Electrophysiological techniques and data analysis. *Xenopus laevis* stage V and VI oocytes were prepared and injected with the appropriate RNA as described by Iverson & Rudy (35). Oocytes were then incubated for 2-3 days at 20-22°C in ND96 (96mM NaCl, 2mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Hepes, pH 7.5) supplemented with penicillin (100 units/ml), streptomycin (100 ug/ml) and 2.5 mM sodium pyruvate. Currents were recorded in ND96 with a two-microelectrode voltage-clamp (35). The membrane potential was monitored during the experiment, and only those experiments where the command voltage was reached and maintained were used. Current dependent artifacts such as electrode polarization or shifts in equilibrium potential are unlikely since the kinetic properties of the various currents shown here are identical independently of the amount of current expressed (35). Microelectrodes were filled with 3M KCl and had input impedances between 1-2MΩ. The pClamp software (Axon Instruments, Foster City, CA) was used to generate pulses and for data analysis. All experiments were carried out at 18-20°C.

Results

Method to study whether *Shaker* subunits form heteromultimeric channels. The strategy used to determine whether distinct Shaker subunits can form heteromultimers relied on co-injection into Xenopus oocytes of two in-vitro transcribed Shaker RNAs differing in their 5′ or 3′ ends. Electrophysiological analysis of the expressed currents is used to assess whether a K+ current, distinct from that obtained when each RNA is injected separately, is obtained. The injection of two different RNAs could result in

the formation of at least three channel types: the two original homomultimeric channels and at least one heteromultimer. The properties of the latter must be sufficiently distinct from those of either homomultimer to confirm that heteromultimeric channels have been formed. This requires the ability to separate each component of the current or to demonstrate that the resulting waveform cannot be due to any algebraic sum of the two currents produced by homomultimers. Because homomultimeric Shaker channels do not differ significantly in their voltage-dependence or pharmacology, it is difficult to separate the currents contributed by individual Shaker channels. Our approach relied on the use of a mutation of the 29-4 Shaker cDNA (35) which expresses channels that are altered in their voltage-dependence (36). In this mutant, (29-4(V2)), the second leucine in the leucine-heptad repeat, conserved among most K+ channels (37), has been changed to a valine. 29-4 (V2) channels are similar, although not identical, to wild type 29-4 channels in their kinetic properties but their voltage-dependence of activation and inactivation is shifted by approximately 70 mV in the depolarizing direction (36, see also Figures 3, 5A and 5B). The nomenclature for Shaker products used here is that of Iverson and Rudy (35). The name of each Shaker product consists of two numbers separated by a dash. The first number indicates the type of 5′ (or amino) end and the second number the type of 3′ (or carboxyl) end.

Formation of heteromultimers of Shaker subunits with two different amino domains. A novel current is expressed in Xenopus oocytes co-injected with two Shaker RNAs differing in their 5′ ends. The analysis of this experiment is shown in Figures 1-3. Figure 1 shows the currents observed in oocytes injected with 37-4 RNA (A), 29-4 (V2) RNA (B), a 1:1 (C), or a 2:1 (D) mixture of both RNAs. 37-4 currents (35) are essentially non-inactivating

except at large membrane depolarizations where the current consists of a small ($\leq$ 10%) transient component and a large non-inactivating component (Figure 1A). These kinetic properties result from the instability of the inactivated state in homomultimeric channels containing type 37 amino domains (35). 37-4 currents, like other Shaker currents, start to activate at membrane potentials between -40 and -30 mV and reach half-maximal conductance at about -10 mV. In contrast, 29-4(V2) channels do not open until the membrane potential reaches about +20 mV (Figure 3). The conductance increases with depolarization less steeply than wild type 29-4 or other Shaker channels and reaches a half-maximal value at about +70 mV (36). Thus, little, if any, 29-4 (V2) current is observed at voltages below +20 mV (Figure 1B). Therefore, if oocytes co-injected with 37-4 and 29-4 (V2) RNAs expressed only homomultimeric channels the resulting current should be identical to 37-4 currents at voltages below +20 mV (i.e., a non-inactivating current). An essentially non-inactivating current is also expected for a simple sum of the two currents at +30 mV, since both, 29-4(V2) and 37-4 currents exhibit little, if any, inactivation at this potential (see Figures 1A, 1B). At more depolarized potentials the sum of the 29-4(V2) and 37-4 currents should be a complicated waveform consisting of both a transient and a steady-state component, depending on the contribution of each current to the total. However, in no case could the transient component be larger than that of 29-4(V2) currents alone since these channels inactivate much more than 37-4 channels (Figures 1A, 1B)

The actual currents obtained in an oocyte injected with a 1:1 mixture of 37-4 and 29-4(V2) are shown in Figure 1C, and a 2:1 mixture in Figure 1D. A transient current is observed at +10 and +30 mV, contrary to what would be expected from a simple sum of the 37-4 and 29-4(V2)currents. Furthermore, at

+50 mV about 70% of the current inactivates, a value larger than that for 29-4(V2) channels at this voltage (40-50%). The currents shown in Figure 1C and 1D leave little doubt that a new channel, i.e., a heteromultimer with properties distinct from either 370-4 or 29-4(V2), is formed. The resulting waveforms can not be any algebraic sum of the original currents.

Given that novel current is observed in oocytes injected with the RNA mixtures, that produced by heteromultimeric channels, we have attempted to separate the currents produced by heteromultimers from those produced by the two homomultimers. Inactivation prepulses, expected to inactivate most of the heteromultimeric channels but few 37-4 or 29-4(V2) channels, were used to isolate the current carried by heteromultimers. An example of this type of analysis for an oocyte injected with a 1:1 mixture of 37-4 and 29-4(V2) RNA, is shown in Figure 2. Currents obtained during test pulses to the indicated potentials following a 500 ms prepulse to -100 mV are shown in Figure 2A or a 500 ms to +20 mV (i.e., the non-inactivated component) in Figure 2B. The difference between the two (i.e. the inactivated component) is shown in Figure 2C. A significant fraction (about 75%) of the total current is inactivated by the +20 mV prepulse (Figure 2C). A quantitative estimate of the contributions made by 37-4 or 29-4(V2) homomultimers to the inactivated component is described in detail in the figure legend. This analysis leads to the conclusion that $\geq$ 93% of the inactivated component consists of heteromultimeric channels. Since $\geq$ 93% of the inactivated component is contributed by heteromultimeric channels we can assume that the properties of this component reflect primarily the properties of these heteromultimers. The conductance-voltage relation (g-V curve) of this component is shown in Figure 3. These channels show a voltage-dependence intermediary between that of 37-4 and 29-4(V2) channels which probably

results from the mixing of leucine-heptad motifs. The kinetics of the heteromultimeric current is closer to that of 29-4 channels. The time course of inactivation of currents carried by *Shaker* channels is determined primarily by the amino domain (35). This suggests that in heteromultimeric channels containing both type 29 and type 37 amino domains, the 29 amino domain, which produces a more stable inactivated state, dominates the kinetics.

Formation of heteromultimers of *Shaker* subunits with two different carboxyl domains. *Shaker* channels with two types of carboxyl domains have been described: type 4 and type 37 (35). The primary effect of the divergent carboxyl domains is to influence the rates of recovery from inactivation of *Shaker* channels, perhaps by influencing the entry into an inactivated state from which recovery is slow (35). All channels with a type 4 carboxyl domain recover fast from inactivation independent of the amino domain. All channels with a type 37 carboxyl domain recover from inactivation extremely slowly.

To demonstrate the formation of heteromultimers of subunits containing different carboxyl domains we have injected oocytes with mixtures of 29-4(V2) (described above) and 29-37 RNAs (35). Because these two RNAs encode proteins with the same amino domain their time courses during depolarizing pulses are similar. However 29-37 channels inactivate almost completely during a depolarizing pulse and recover very slowly from inactivation (35), while 29-4(V2) channels show incomplete inactivation (Figure 1B) and fast recovery from inactivation (Figure 5C). Like other *Shaker* currents, 29-37 channels start to activate at membrane potentials between -40 and -30 mV and reach their half-maximal conductance at about -10 V, and like all inactivating *Shaker* channels are fully inactivated at about -10 mV (35). By taking advantage of the differences in the voltage-dependence of

inactivation of 29-37 and 29-4(V2) channels we have been able to separate the currents observed in oocytes co-injected with these RNAs (Figure 4). The total current obtained in an oocyte co-injected with both RNAs is shown in Figure 4A. The currents observed following a 1 sec prepulse to -10 mV (the non-inactivated component) are shown in Figure 4B. Figure 4C shows the difference between the two, i.e., the inactivated component.

The 1 sec prepulse to -10 mV inactivates about 70% of the current shown in Figure 4A. This prepulse should inactivate all 29-37 but none of the 29-4(V2) homomultimers (35, 36, Figure 5B). If no heteromultimeric channels are formed in an oocyte injected with both RNAs the non-inactivated component should only consist of 29-4(V2) currents. However, as shown in Figure 4B, a measurable amount of current is detectable in the non-inactivated component at voltages below those that activate 29-4(V2) channels. Furthermore, the currents decay more during a pulse than 29-4(V2) currents alone (Figure 1B). Therefore the non-inactivated component must consist, at least in part, of a novel current carried by heteromultimeric channels. The inactivated component (Figure 4C) is similar to 29-37 currents (35) in that they inactivate almost completely during a depolarizing pulse. The g-V curve for this component is also similar, but not identical, to that of 29-37 (Figure 5A). The differences between the two g-V curves suggest that some heteromultimeric channels are also inactivated by the prepulse to -10 mV. It is possible that the heteromultimeric component may contain more than one type of heteromultimeric channel. The conductance-voltage curve of the non-inactivated component (Figure 5A) which may consist of both heteromultimeric and 29-(V2) homomultimeric channels, lies between that of 29-37 and 29-4(V2) channels, and is similar to that of 37-4 + 29-4(V2) heteromultimers (Figure 3). The steady-state inactivation properties of the

non-inactivated component (Figure 5B) indicate that most of it is derived from heteromultimeric channels; the current is 80% inactivated by a prepulse to +20 mV a voltage which inactivates only about 20-30% of 29-4(V2) currents (Figure 5B). Thus, at most 38% (30%/80%) of the non-inactivated component can be contributed by 29-4(V2) channels. The conductance of 29-4(V2) channels at +20 mV is five-fold less than that at +40 mV (36; Figure 3 in Chapter 3 of this thesis). The fact that the steady-state inactivation curves of the non-inactivated component are similar with test pulses to +20 or +40 mV (Figure 5B) suggests that the fraction of 29-4(V2) channels in the non-inactivated component must be much less than 38%. From this analysis we conclude that most (>> 62%) of the non-inactivated current shown in Figure 4B is carried by heteromultimeric channels. Recovery from inactivation of 29-37, 29-4(V2) and the presumed heteromultimeric channels are shown in Figure 5C. Recovery of the non-inactivated component is similar at test pulses of +20 (data not shown) or at +40 mV, again supporting the conclusion that the contribution of 29-4(V2) channels to the non-inactivated component is small. Although these heteromultimeric channels recover from inactivation at rates intermediate to those of 29-37 and 29-4(V2) homomultimers, the rate is more similar to that of 29-4(V2) channels. This indicates that the kinetics of recovery from inactivation of heteromultimers is more similar to homomultimeric channels containing the carboxyl domain that produces the faster recovery kinetics.

Lack of formation of functional heteromultimeric channels between two subunits of different classes of *Sh* family genes. To test whether products of *Sh* family genes belonging to distinct classes, and hence with more divergent sequences, can also form heteromultimers with novel functional properties we co-injected *Shaker* 29-4 RNA (35) with RKShIIIA RNA.

RKShIIIA was cloned from a rat brain cDNA library (23), and is more similar to the *Drosophila Shaw* gene than to to *Shaker* (14). cRNA derived from RKShIIIA expresses voltage-dependent delayed-rectifier type K+ channels which activate very slowly and at large membrane depolarizations (23). The currents observed in oocytes injected with mixtures of 29-4 and RKShIIIA RNAs appear to be an algebraic sum of the currents observed when each RNA is injected separately (Figure 6). The currents in an oocyte injected with a 1:1 mixture of 29-4 and RKShIIIA contain an initial transient component followed by a late, slowly rising, non-inactivating component (Figure 6A & B). A prepulse which inactivates 100% of 29-4 currents, but no RKShIIIA currents, separates the currents in co-injected oocytes into two components. The component not-inactivated by the prepulse is kinetically similar, and has the same voltage-dependence as RKShIIIA currents alone (Fig. 6 C, D, G). On the other hand, the inactivated component, obtained by subtraction, is kinetically similar, and has the same voltage-dependence as 29-4 currents alone (Fig. 6 E, F, H).

RKShIIIA currents are very sensitive to the K+ channel blocker tetraethylammonim (TEA) and are nearly completely blocked at 1 mM (23). In contrast, 29-4 currents are only partially blocked by much higher concentrations of TEA (35). TEA also separates the currents observed in oocytes injected with mixtures of 29-4 and RKShIIIA into a 29-4-like and a RKShIIIA-like current (data not shown). The failure to observe any significant amount of current with obvious different properties in oocytes co-injected with 29-4 and RKShIIIA RNAs suggests that the products of these RNAs cannot form heteromultimers. It is unlikely that this is a result of species differences, since, *Shaker* subunits (including 29-4) form

heteromultimeric channels when mixed with rat homologs of the same class (33).

Discussion

Subunit recognition by *Sh* gene products. We show here that heteromultimers can be formed from *Shaker* subunits which differ in the amino or in the carboxyl termini, indicating that these variable domains do not prevent subunit interactions between divergent *Shaker* gene products. Subunit interactions involved in channel assembly may therefore depend primarily on amino acid sequences that are similar to all *Shaker* proteins. Mammalian homologues of *Shaker* (Class 1 *Sh* family genes), also form functional heteromultimers: among themselves (31, 34) or with *Drosophila Shaker* products (33). These proteins are very similar in a core region which starts about 130 residues before the first putative membrane spanning domain and terminates a few residues after the sixth putative membrane spanning domain. In this region, these proteins show about 70% amino acid identity with most of the differences found in the three linkers between the first four putative membrane spanning domains. On the other hand we show here data that suggests that *Shaker* subunits (29-4) do not form functional heteromultimers with RKShIIIA. These proteins differ more in the core region. RKShIIIA (a class III *Sh* family gene) shows only about 40% identity in this region with *Shaker* and its rat homologs (23). Taken together the data indicates that sequences responsible for subunit recognition might be found somewhere in the core region in sequences that are similar among all Class I genes and are different between Class I and Class III subunits.

Heteromultimer formation and K+ channel diversity. Based on the finding that the products of two different classes of *Sh* family genes: 29-4 (Class I) and RKShIIIA (Class III), do not form functional heteromultimers the possibility should be considered that subunits of certain classes do not mix. Indeed, Wei et al., (24) have indicated that *Shaw* and *Shal* (Class III & IV), and *Shab* and *Shal* (Class II & IV) do not seem to form heteromultimers, and have suggested that these classes of *Sh* family genes represent independent channel systems. Our results are consistent with the idea that some classes of *Sh* family genes may behave physiologically as independent channel systems.

Our results show how novel subtypes of K+ channels, with diverse voltage-dependent and kinetic properties, can be generated by the formation of heteromultimers. Heteromultimer formation between similar subunits might be responsible for the subtle differences in the properties of a given K+ channel type observed in closely related cells. For example, two recent studies on A-type K+ currents in molluscan showed a variety of inactivation kinetics between similar neurons in different ganglia (40), as well as between different neurons within the same ganglion (41). Combinations of different subunit types has also been suggested as a mechanism to generate functional diversity of $GABA_A$ receptors (42). Heteromultimer formation may also be responsible for the generation of novel K+ channel types. For example, in spite of the large number of voltage-dependent K+ channel genes cloned from mammalian brain, and expressed in *Xenopus* oocytes and other expression systems, none of these channels appear to correspond to some of the most typical voltage-dependent K+ channels observed in many neurons: the typical delayed-rectifier channel that has been recorded in the cell bodies of several neurons (reviewed in 4, 5) that is TEA sensitive and 4-AP insensitive; the

typical low-threshold "A" current (4, 43); and the "M" channel (4). These channels may be encoded by genes that have not yet been cloned. Alternatively, the expression systems used, in particular the *Xenopus* oocyte system, may not be capable of forming some of these channels because of differences in posttranslational modifications. The results described here suggest a third possibility: that some of these typical K+ channels may only be produced by heteromultimer formation. The expression of these channels in oocytes could therefore, depend on co-injection of a particular combination of two or more different RNAs.

Since the properties of K+ channels in a given cell may depend, among other factors, on both the number and type of associated subunits (see also 38, 39) complex regulatory mechanisms may be in effect at the level of expression, subcellular localization and assembly. Given these complexities it may not be surprising to K+ channel subtypes that may be expressed in only a few cell types. In addition, it may be difficult to correlate the functional types of K+ channels in a particular cell with the subunits expressed in that cell. In this context, our results would establish some of the rules that determine the kinetic properties of heteromultimeric channels. Specifically for subunits with identical carboxyl ends the kinetics of heteromultimers is closer to that produced by the subunit with the amino end that produces more inactivation. Heteromultimers with different carboxyl termini exhibit a rate of recovery from inactivation that is more similar to that produced by the fast recovering subunits. Furthermore the mixing of leucine-heptad motifs leads to the formation of channels with intermediary voltage-dependence.

Expression of *Shaker* heteromultimers in-vivo. It is possible that not all combinations of subunits that are allowed in *Xenopus* oocytes occur in-vivo. For example, particular *Shaker* subunits may may be localized to

specific subcellular regions and would therefore not be able to participate in heteromultimer formation. However, recent results suggest that the A_1 current expressed in Drosophila muscle (44) may be a heteromultimer of *Shaker* gene products. The Sh^{E62} allele results in a large reduction in the amount of A1 current in larval muscle (45). Analysis of this mutation indicates a sequence difference in, or near, the splice junction for the type 37 3' end (46), suggesting that this mutant may be incapable of expressing subunits with type 37 carboxyl domains. It is unlikely that the native muscle channel is multimer containing only type 37 carboxyl domains since currents produced by these channels in oocytes exhibit extremely slow recovery from inactivation and such currents are not observed in *Drosophila* muscle (47, 48). Our results indicate that heteromultimers containing both type 4 and type 37 carboxyl ends produce currents with fast recovery from inactivation. Thus, the A1 current in Drosophila muscle may be carried by these heteromultimers. Furthermore, none of the homomultimeric channels expressed in oocytes exhibit properties that are identical to the native muscle A1 channel (28). Germ line transformation experiments indicate that the kinetic properties of homomultimeric 4-4 channels are identical to those in *Xenopus* oocytes (49), but different than those in native muscle. Namely they inactivate and recover from inactivation faster than the native channel. Given our results the combination of a type 4 and a type 37 carboxyl domain could account for the differences. Taken together, these results suggest that the native muscle A1 channel is formed by association of different subunits, as genetic experiments in *Drosophila* suggested (45, 50, 51).

Acknowledgements

This research was supported by U.S. Public Health Service Grants GM26976 to BR; NS28135 to LI and NS21327 to Mark A. Tanouye.

References

1. Dubois, J.M. (1983) *Prog. Biophys. Molec. Biol.* **42**, 1-20

2. Hille, B. (1984) *Ionic Channels of Excitable Membranes*, (Sinauer, MA).

3. Llinas, R. (1988) *Science* **242**, 1654-1664

4. Adams, P.R. & Galvan, M. (1986) In *Advances in Neurology* Vol. **44** (A. Delgado-Esueta, A.A. Ward, D.M. Woodbury & R. Porter eds.) Raven, NY.

5. Rudy, B. (1988) *Neuroscience* **25**, 729-750

6. Lewis, R.S. & Cahalan, M.D. (1988) *TINS* **11**, 214-218

7. Tempel, B., Papazian, D., Schwarz, T., Jan Y., & Jan, L.Y. (1987) *Science* **237**, 770-775

8. Kamb, A., Iverson, L.E., & Tanouye, M.A. (1987) *Cell* **50**, 405-413

9. Schwarz, T., Tempel, B., Papazian, D., Jan Y., & Jan, L.Y. (1988) *Nature* **331**, 137-142

10. Pongs, O., Kecskemethy, N., Muller, R., Krah-Jentgens, I., Baumann, A., Klitz, H., Canal, I., Llamazares, S., & Ferrus, A. (1988) *EMBO J.* **7**, 1087-1096

11. Kamb, A., Tseng-Crank, J., & Tanouye, M.A. (1988) *Neuron* **1**, 421-430

12. Tempel, B., Jan Y., & Jan, L.Y. (1988) *Nature* **332**, 837-839

13. McKinnon, D. (1989) *J. Biol. Chem.* **264**, 8230-8236

14. Butler, A., Wei, A., Baker, K. & Salkoff, L. (1989) *Science* **243**, 943-947

15. Stuhmer, W., Ruppersburg, J.P., Schroter, K., Sakmann, B., Stocker, M., Giese, K., Perschke, A., Baumann, A. & Pongs, O. (1989) *EMBO J.* **8**, 3235-3244

16. Frech, G.C., VanDongen, A.M.J., Schuster, G., Brown, A.M. & Joho, R.H. (1989) *Nature* **340**, 642-645

17. Kamb, A., Weir, M., Rudy, B., Varmus, H. & Kenyon, C. (1989) *Proc. natn. Acad. Sci. USA* **86**, 4372-4376

18. Christie, M.J., Adelman, J.P., Douglass, J. & North, A.J. (1989) *Science* **244**, 221-224

19. Yokoyama, S., Imoto, K., Kawamura, T., Higashida, H., Iwabe, N., Miyata, T. & Numa S. (1989) *FEBS Lett.* **259**, 37-42

20. Swanson, R., Marshall, J., Smith J., Williams, J.B., Boyle, M.B., Folander, K., Luneau, C.J., Antanavage, J., Oliva, C., Buhrow, S.A., Bennett, C., Stein, R.B., & Kaczmarek, L. (1990) *Neuron* **4**, 929-939

21. Tseng-Crank, J., Tseng, G.N., Schwartz, A. & Tanouye, M.A. (1990) *FEBS Lett.* in press

22. Ramaswami, M., Gautam, M., Kamb, A., Rudy, B., Tanouye, M.A. & Mathew, M.K. (1990)*Cell. Mol. Neurosci.* in press

23. McCormack T., Vega-Saenz de Miera, E. & Rudy, B. (1990c) *Proc. natn. Acad. Sci. USA* **87**, 5227-5231

24. Wei, a., Covarrubias, M., Butler, A., Baker, K., Pak, M., & Salkoff, L. (1990) *Science* **248**, 599-603

25. Iverson, L.E., Tanouye, M.A., Lester, H., Davidson, N. & Rudy, B. (1988) *Proc. natn. Acad. Sci. USA* **85**, 5723-5727

26. Timpe, L.C., Schwarz, T., Tempel, B., Papazian, D., Jan Y., & Jan, L.Y. (1988a) *Nature* **331**, 143-145

27. Timpe, L.C., Jan Y., & Jan, L.Y. (1988b) *Neuron* **1**, 659-667

28. Zagotta, W.N., Hoshi. T., & Aldrich, R.W. (1989a) *Proc. natn. Acad. Sci. USA* **86**, 7243-7247

29. Leonard, R., Karschin, A., Aiyar, J., Davidson, N., Tanouye, M.A., Thomas, L., Thomas, G., & Lester, H. (1989) *Proc. natn. Acad. Sci. USA* **86**, 7629-7633

30. Gisselman, G., Sewing S., Madsen, B.W., Mallart, A., Anguat-Petit, D., Muller-Holtkamp, F., Ferrus, A. & Pongs, O. (1989) *EMBO J.* **8**, 2359-2364

31. Christie, M.J., North, A.R., Osborne, P.B., Douglass, J. & Adelman, J.P. (1990) *Neuron* **4**, 405-411

32. McCormack, K., Lin, J.W., Ramaswami, M., Tanouye, M.A., Iverson, L.E. & Rudy, B. (1990a) *Biophys. J.* **57**, 209a

33. Isacoff, E.Y., Jan, Y.N. & Jan, L.Y. (1990) *Nature*, **345**, 530-534

34. Ruppersburg, J.P., Schroter, K.H., Sakmann, B., Stocker, M., Sewing, S. & Pongs, O. (1990) *Nature* **345**, 535-537

35. Iverson, L.E. & Rudy, B. (1990) *J. Neurosci.* **10**, 2903-2916

36. McCormack, K., Tanouye, M.A., Iverson, L.E., Lin, J.W., Ramaswami, M., McCormack, T., Campanelli, J.T., Mathew, M.K. & Rudy, B. *Proc. natn. Acad. Sci. USA*, in press (Thesis-Chapter 3)

37. McCormack, K., Campanelli, J.T., Ramaswami, M., Tanouye, M.A., Iverson, L.E. & Rudy, B. (1989) *Nature* **340**, 103

38. Rudy, B., Hoger, J., Lester. H. & Davidson, N. (1988) *Neuron* **1**, 649-658

39. Rehm, H. & Lazdunski, M. (1988) *Proc. natn. Acad. Sci. USA* **85**, 4919-4923

40. Serrano, E.E. & Getting, P.A. (1989) *J. Neurosci.* **9**, 4021-4032

41. Premack, B., Thompson, S. & Coombs J. (1989) *J. Neurosci.* **9**, 4089-4099

42. Levitan, E., Schofield, P., Burt, D., Rhee, L., Wisden, W., Kohler, M., Fujita, N., Rodriguez, H., Stephenson, A., Darlison, M., Barnard, E. & Seeburg, P. (1988)*Nature*, **335**, 76-79

43. Segal, M., Rogawski, M. & Barker, J.L. (1984) *J. Neurosci.* **4**, 604-609

44. Solc, C., Zagotta, W.N. & Aldrich, R.W. (1987) *Science* **236**, 1094-1098

45. Haugland, F.N. & Wu, C.F. (1990) *J. Neurosci.* **10**, 1357-1371

46. Pongs, O., Muller, R., Lichtinghagen, R., Sewing, F., Stuhmer, W., Stocker, M., Gisselman, G. & Ferrus, A. (1989) *Drosophila molecular neurobiology Cold Spring Harbor meeting Abstracts* 145.

47. Salkoff, L. (1983) *Cold Spring Harbor Symp. Quant. Biol.* **XLVIII**, 221-231

48. Wu, C.F. & Haugland, F.N. (1985) *J. Neurosci.* **5**, 2626-2640

49. Zagotta, W.N., Germeraad, S., Garber, S., Hoshi, T. & Aldrich, R.W. (1989b) *Neuron* **3**, 773-782

50. Haugland, F.N. & Wu, C.F. (1986) *Biophys, J.* **49**, 168a

51. Timpe, L.C., Jan, Y.N. & Jan, L.Y. (1987)*J. Neurosci.* **7**, 1307-1317

Figure 1. K^+ currents in *Xenopus* oocytes injected with *Shaker* RNAs with variable 5′ ends. Currents obtained during a series of depolarizing pulses from -70 mV to +50 mV in 20 mV increments given at a frequency of one every 8 sec in oocytes injected with 37-4 RNA (A), 29-4(V2) RNA (B), or a 1:1 (C), or a 2:1 (D) mixture of 37-4 and 29-4(V2) RNAs. The membrane potential was held at -100 mV between pulses. Vertical calibration: 990 nA (A); 1700 nA (B); 1400 nA (C); 390 nA (D). Horizontal calibration 16 ms.

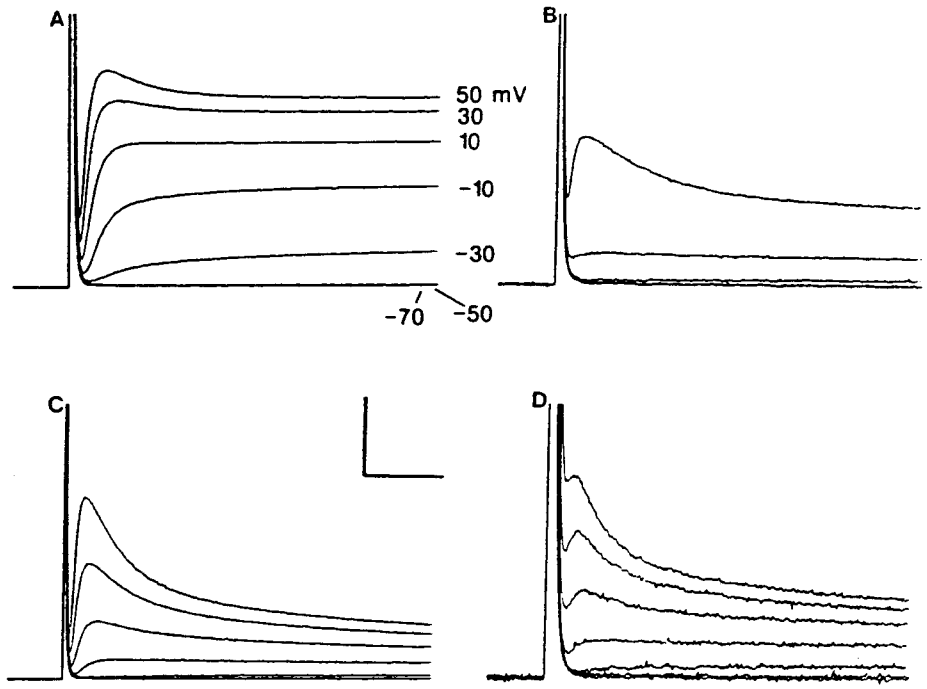

A
B
50 mV
30
10
-10
-30
-70
-50
C
D

Figure 2. Separation of current components in an oocyte injected with a 1:1 mixture of 37-4 and 29-4(V2) RNA. (A) The oocyte was held at -100 mV for 500 ms and then stepped to the indicated test potentials. (B) The oocyte was held at +20 mV for 500 ms and then stepped to the same potentials as in part A. In both A and B pulses were given once every 8 sec and the membrane was held at -100 mV between pulses. (C) Currents obtained by subtracting the currents in part B from those in part A. In all cases only the currents obtained during test pulses are shown.

The contributions of homo and heteromultimers to the inactivated component (C) was calculated as follows: An upper limit estimate of the current contributed at any voltage by 37-4 channels can be obtained from the current at -30 mV and its known g-V curve (35), assuming the current contributed by 29-4(V2) or heteromultimeric channels at this potential is negligible. The current at -30 mV is about 40 nA for the experiment shown in Figure 2A which gives a conductance of 0.7×10^{-6}S assuming a reversal potential of -90 mV. Since roughly 10-12 % of the 37-4 conductance is activated at -30 mV (35), the 37-4 current at +50 mV (maximal conductance) should be 750 to 930 nA. Because the peak current amplitude of the total current at +50 mV is about 3860 nA, 37-4 channels contribute at most 24% (390/3860) of this current. The remaining $\geq$ 76% of the total current at +50 mV must therefore be contributed by 29-4(V2) and heteromultimeric channels. A 500 ms prepulse to +20 mV inactivates about 10% of 37-4 currents (primarily the transient component: data not shown) observed at large depolarizing potentials. If at most 24% of the total current at +50 mV is contributed by 37-4 homomultimeric channels, then a prepulse to +20 mV should remove only about 2.4% of this current. This suggests that although 75% of the total

current is inactivated by the prepulse to +20 mV, only about 3.2% (2.4%/75%) of the inactivated current (2C), at +50 mV, can be contributed by 37-4 channels. At less positive voltages its relative contribution should be even smaller.

We estimated above that the total contribution of 37-4 channels at +50 mV was about 750-930 nA. Most ($\geq$ 90 %) of the 37-4 current should be present in the non-inactivated component. This represents 71-88% (.9 x [750-930 nA]/the non-inactivated current at +50 mV = 950 nA) of the non-inactivated current at +50 mV in Figure 2B. Therefore no more than 29% of the non-inactivated current can contributed by 29-4(V2) and heteromultimeric channels. If we assume that most heteromultimeric channels are inactivated by the prepulse to + 20 mV, then the current carried by 29-4(V2) channels in the non-inactivated component at + 50 mV can be no more than 275 nA (29% of the peak non-inactivated current at +50 mV = 950 nA). From 29-4(V2) inactivation curves (see Figure 5B) it is known that a +20 mV prepulse inactivates about 20-30% of this current. Therefore the contribution of the 29-4(V2) current to the total current is, at +50 mV, at most 393 nA (275/.7). This accounts for 10% (393/peak total current at +50 mV= 3860 nA) of the total current at +50 mV. Given the voltage-dependence of 29-4(V2) channels, at less positive voltages, its relative contribution should be even smaller. We can now calculate the maximum contribution of 29-4(V2) channels to the inactivated component shown in Figure 2C. If $\leq$ 10.0% of the total current is 29-4(V2), and 20-30% of this current is inactivated by a 500 ms prepulse to +20 mV, the inactivation of 29-4(V2) channels should result in inactivation of at most 3% (10% of 30%) of the total current in Figure 2A. Therefore, 29-4(V2) channels could contribute at most 4% (3% / the percentage of inactivated current = 75%) of the current in the inactivated component (Figure 2C).

Hence, less than 7.2% (3.2% + 4%) of the inactivated component is contributed by homomultimeric channels, and $\geq$ 93% by heteromultimers.

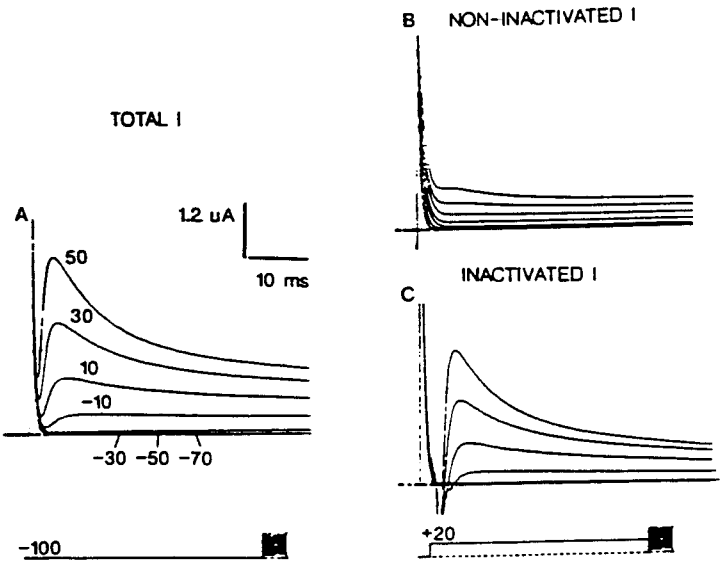

TOTAL I
A
1.2 uA
10 ms
50
30
10
-10
-30 -50 -70
-100
B NON-INACTIVATED I
INACTIVATED I
C
+20

Figure 3. Comparison of the voltage-dependence of homomultimeric and heteromultimeric *Shaker* channels. Relative peak conductance is plotted against membrane potential. The relative peak conductance was obtained by dividing the conductance at the indicated voltage ($G=I/V-V_k$) by the maximal conductance (G_{max}). The conductance was calculated assuming a reversal potential of -90 mV. Relative conductance of *Shaker* currents obtained in an oocyte injected with 37-4 RNA (filled triangles), 29-4(V2) RNA (filled circles), and the inactivated component of the experiment shown in fig. 2 (open squares).

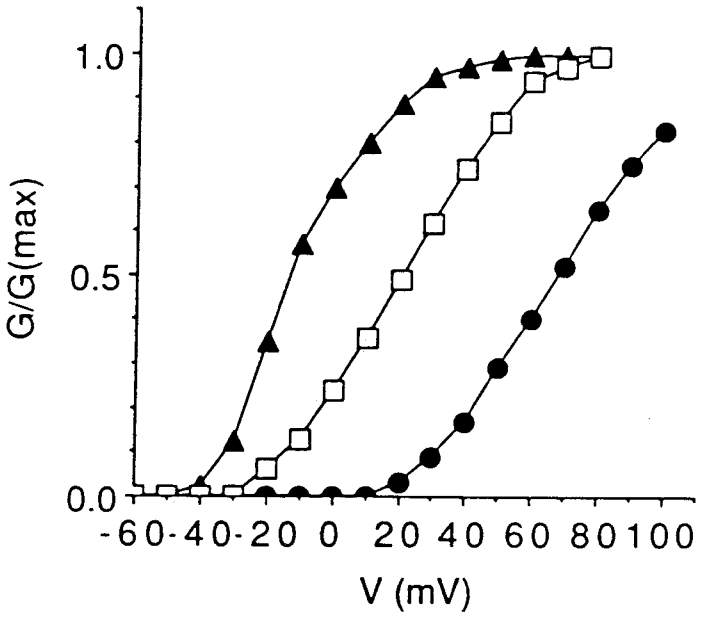

1.0
0.5
0.0
G/G(max)
-60 -40 -20 0 20 40 60 80 100
V (mV)

Figure 4. Separation of current components in an oocyte co-injected with 29-37 and 29-4(V2) RNAs. (A) The oocyte was held at -100 mV for 1 sec and then stepped to the indicated test voltages. (B) The oocyte was held at -10 mV for 1 sec and then stepped to the same voltages as in part A. In both A and B the pulses were given once every 30 sec and the membrane was held at -100 mV between pulses. (C) Currents obtained by subtracting the currents in part B from those in part A. In all cases only the currents obtained during the test pulses are shown.

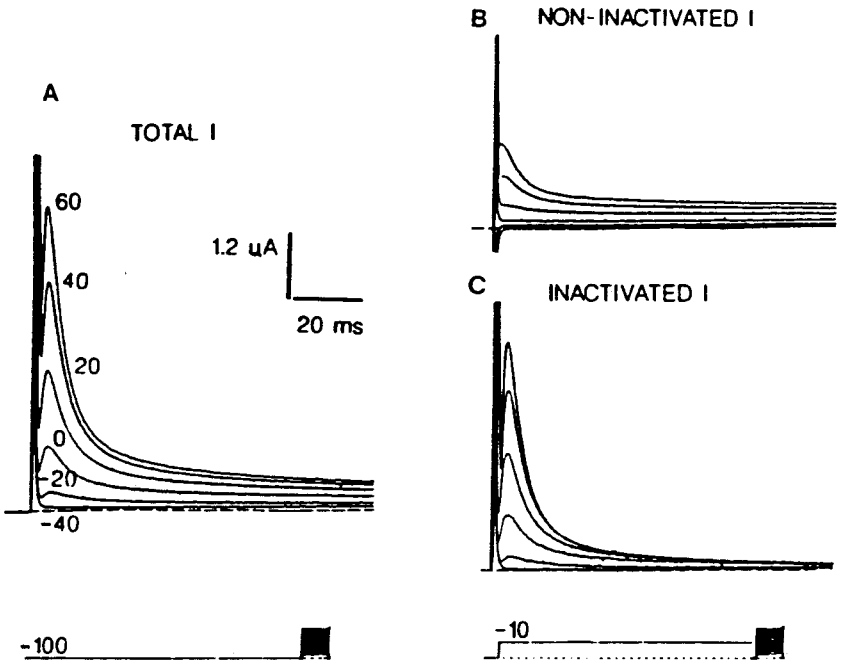

A
TOTAL I
60
40
20
0
-20
-40
1.2 uA
20 ms
-100
B
NON-INACTIVATED I
C
INACTIVATED I
-10

Figure 5. Properties of heteromultimeric channels with different carboxyl domains. (A) Conductance-voltage relation for the inactivated component shown in Figure 4C (open squares); the non-inactivated component shown in Figure 4B (open circles); and 29-37 (filled squares), and 29-4(V2) (filled triangles) currents obtained from oocytes injected with the corresponding single RNA species. Relative conductance, determined as in Figure 3, is plotted as a function of membrane potential. (B) Steady-state inactivation of the inactivated component (open squares); the non-inactivated component (open circles) 29-37 (filled boxes) and 29-4(V2) currents (filled triangles). The membrane potential was held at -100 mV followed by 1 sec prepulses to the voltages indicated in the abscissa and then stepped to +40 mV. Because the inactivated component is defined as that one that is completely inactivated by prepulses to -10 mV, the current obtained during the test pulse following the 1 sec prepulse to -10 mV was defined as the zero value for this component. Current amplitudes for the inactivated component {I(V)} were therefore obtained by subtracting this zero value from the actual current amplitudes. I_{max} for the inactivated component was defined as the current amplitude obtained during the test pulse following a prepulse to -10 mV subtracted from the current amplitude obtained following the -60 mV prepulse. For the non-inactivated component I_{max} was defined as the current amplitude following the -10 mV prepulse. Relative current amplitudes for 29-4(V2) and 29-37 currents, alone, were calculated by dividing the current obtained following prepulses to the indicated voltages by the current obtained following the -60 mV prepulse, with no subtraction. Small open circles are data for the non-inactivated component with test pulses to +20 mV. (C) Recovery from inactivation for the non-inactivated component (open circles); 29-37 (filled

boxes); and 29-4(V2) currents (filled triangles). Two identical pulses separated by the indicated intervals were applied. The peak current obtained during the second test pulse, divided by the peak current obtained during the first test pulse, is plotted as a function of the interval between the two test pulses. For the 29-4(V2) and the 29-37 currents, the holding potential was -100 mV and test pulses were to +40 mV. For the non-inactivated component each pulse consisted of a 1 sec prepulse to -10 mV followed by a test pulse to +40 mV.

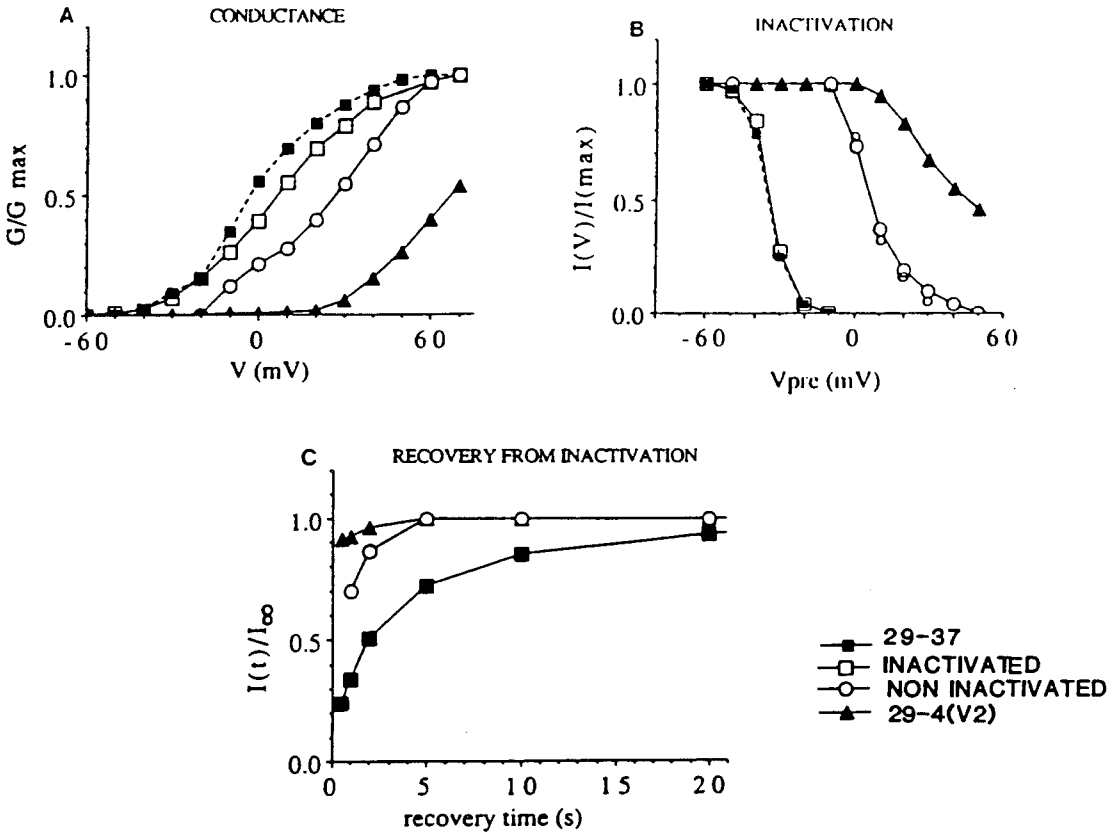
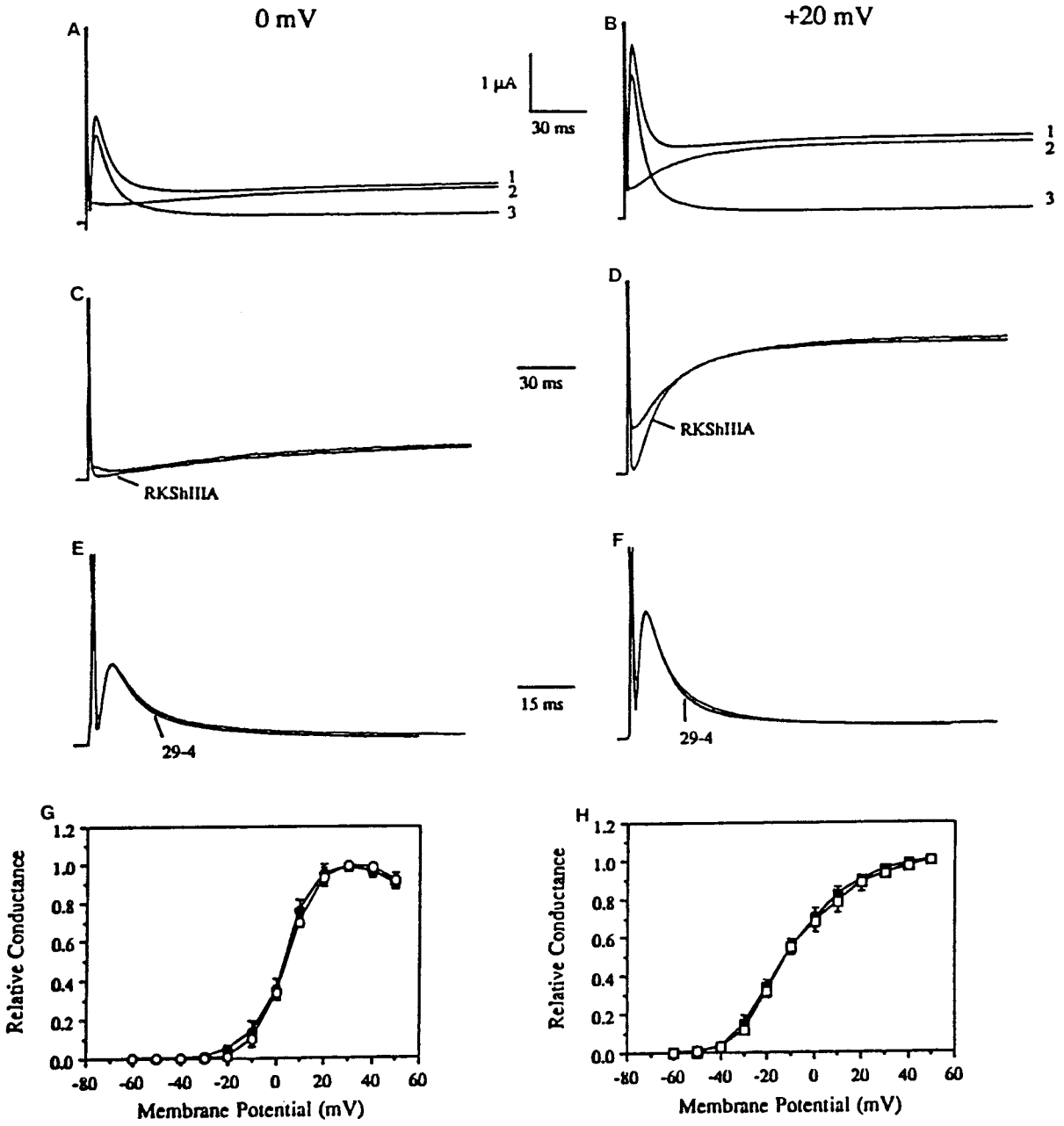

A
CONDUCTANCE
G/G max
1.0
0.5
0.0
-60
0
60
V (mV)
B
INACTIVATION
I(V)/I(max)
1.0
0.5
0.0
-60
0
60
Vpre (mV)
C
RECOVERY FROM INACTIVATION
I(t)/I∞
1.0
0.5
0.0
0
5
10
15
20
recovery time (s)
29-37
INACTIVATED
NON INACTIVATED
29-4(V2)

Figure 6. Currents in oocytes injected with *Sh* RNAs of two distinct classes. **A & B**: Currents in an oocyte injected with a 1:1 mixture of 29-4 and RKShIIIA RNAs. Three traces are shown in each panel: (1) Currents during a depolarizing pulse to the indicated voltage following a 1 sec prepulse to -100 mV. (2) Currents during a depolarizing pulse to the indicated voltage following a 1 sec prepulse to -20 mV. (3) The difference between trace 1 and 2. **C & D**: Comparison of the non-inactivated component (trace 2) in A (C) or B (D) with the currents observed at the same voltage in an oocyte injected with RKShIIIA alone. For purposes of comparison the traces have been scaled so that they reach the same peak height and offset so that the baselines are the same. The same scaling and offset, however, was used for C & D. **E & F**: Comparison of the inactivated component (trace 3) in A (E) or B (F) with the currents observed at the same voltage in an oocyte injected with 29-4 RNA alone. For purposes of comparison the traces have been scaled so that they reach the same peak height and offset so that the baselines are the same. The same scaling and offset. however, was used for E & F. **G**: Comparison of the normalized g-V curve, obtained as in Figure 3 but utilizing the current at 250 ms, of the non-inactivated component in experiments such as those shown in A & B (filled circles) with the g-V curve of RKShIIIA currents (open circles). Shown are averages of 3 experiments ± SD. Curve drawn by eye. **H**: Comparison of the normalized g-V curve, determined as in Figure 3, of the inactivated component in experiments such as those shown in A & B (filled squares) with the g-V curve of 29-4 currents (open squares). Shown are averages of 3 experiments ± SD. Curve drawn by eye.


A
0 mV
1 µA
30 ms
1
2
3
B
+20 mV
1
2
3
C
RKShIIIA
30 ms
D
RKShIIIA
E
29-4
15 ms
F
29-4
G
Relative Conductance
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-80 -60 -40 -20 0 20 40 60
Membrane Potential (mV)
H
Relative Conductance
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-80 -60 -40 -20 0 20 40 60
Membrane Potential (mV)

Chapter 4

Discussion

Structure-Function Relationships of *Shaker* K+ channels

The functional and structural correlates of voltage-dependent ion channels has recently received much attention. Intensive investigation of *Sh* and other *Sh* family members has revealed many of the physiologically significant regions in these K+ channels. These studies have focused on the structural determinants of pharmacology, conductance, inactivation, subunit assembly and voltage-dependent activation. The results are now discussed in the context of the recent literature.

Conductance and the Channel Pore. Site-directed mutagenic analyses of *Sh* channels, in combination with these pharmacological agents, have revealed an external region of the pore. Mutations of charged residues in the proposed external regions of *Sh* were made to determine their effect on CTX sensitivity (charged residues were substituted because of the electrostatic binding sensitivity of CTX). Substitutions of residues around the H5 domain altered the sensitivity of the channel currents to CTX (1). (There is some controversy as to the active ingredient in their CTX preparation.) This region, which is highly conserved among the different K+ channels, has been proposed to form part of the external pore region in some structural models of K+ and Na+ channels (2). The mutation of residues in other proposed internal and external regions of *Sh* did not alter CTX sensitivity (3). More recently, it has been shown that the substitution of a specific threonine residue to arginine, T449R, (or glutamine or lysine) alters CTX block; it also alters the sensitivity of the channel to block by TEA and the single-channel conductance properties of *Sh* channels (4). A substitution of the same residue,

T449R, may occur naturally in some *Drosophila Sh* products through alternative splicing (see Appendix). In Na+ channels, TTX binding has been studied as well. A mutation which affected TTX binding also altered single-channel conductance (5). Thus, an external portion of the pore appears to reside in the H5 region for the superfamily of voltage-dependent ion channels.

The region which determines the channel pore on the internal side of the membrane is less clear. In the proposed topology of *Sh* , there are only two probable cytoplasmic regions that might act as the internal mouth of the pore. One of these, the region between the proposed transmembrane segments S2 and S3, is not well conserved among the family of *Sh* channels (not shown). The other region lies between the proposed transmembrane segments S4 and S5, and contains the conserved leucine-heptad repeat. Characterization of mutations in this region shows that it contains some of the properties expected for determining an internal pore region (Chapter 2B, ref. 6). In addition, the single channel conductance of some of the heptad mutants may be altered (Lin, J.W. and Rudy, B. unpublished results). Thus, the heptad-repeat region probably forms an internal portion of the channel pore.

Inactivation. *In-vitro* synthesized RNA of the *Sh* splice products, when injected into *Xenopus* oocytes, produce transient "A" type K+ currents that open within a few ms in a voltage-dependent manner, and then inactivate (but see below). The inactivation properties are similar to those seen in native muscle and cultured myotubes (7-11). Analysis of a larger collection of *Sh* splice products has shown that *Sh* can code for inactivating, as well as noninactivating, channels (12). Iverson and Rudy have examined the largest collection of splice products and their analysis suggests that the 5′ splice

product or amino end determines the rate of inactivation, while the 3′ splice product or carboxyl end determines the rate of recovery from inactivation. *Sh* homologs in rat (13) and human (14) also code for a range of inactivating and noninactivating channels. Thus, the physiological classification of transient "A" type and the noninactivating delayed-rectifier type K+ channels (15) has become somewhat blurred by the graded properties of these channels.

One exception to the Hodgkin and Huxley model (see Introduction) is the independence of the inactivating particle. This has been shown to be inaccurate for inactivating Na+ (16, 17) and K+ channels (18). Rather, inactivation appears to be coupled to activation; most channels do not inactivate until they have opened. An unlikely structural model for the inactivation process of Na+ channels was suggested by Armstrong (19). In this model, the inactivation particle, a portion of the protein pictured as a "ball," was attached to the rest of the protein by a "chain;" the ball swings around as a function of the chains ability to undergo conformational transitions. In the open state the ball binds to a receptor site in the mouth of the pore, thereby blocking it. This model was recently examined at the single channel level for the inactivation mechanism of *Sh* channels (20); application of trypsin to the intracellular side of the membrane eliminated the inactivation process of these channels in a similar manner to that seen for Na+ channels over a decade ago (21). Deletions of as few as three *Sh* N terminal amino acids (residues 6-9) gave similar results. This cytoplasmic region was suggested to act as the ball. In addition, deletions and insertions downstream of this region resulted in increases and decreases in the rates of inactivation, respectively. It was suggested that the effect of these mutations could be explained in terms of shortening and lengthening of the chain; shortening should increase, and lengthening should decrease, the rates of inactivation. Deletions of the

N-terminal region of inactivating vertebrate *Sh* homologs also results in a loss of inactivation (14).

In a further study by Aldrich and coworkers, a peptide corresponding to the first 20 amino acids of the N-terminal of a particular *Sh* product (*Sh* B), was applied to the internal surface of membranes containing noninactivating N- terminal deleted mutants (22). Peptide application resulted in channel inactivation in a concentration dependent manner, with a time course similar to the inactivation seen in wild type *Sh* B channels. Peptides with amino acid substitutions did not result in inactivation of these channels. Furthermore, application of the *Sh* B peptide to a noninactivating rat *Sh* homolog resulted in inactivation at similar concentrations and time course to *Sh* B. Thus, the receptor site for this peptide must be conserved between these two proteins; the leucine-heptad repeat region is well conserved in these channels (Ch. 2B fig. 1, ref. 6), suggesting that it might act as the "ball receptor."

The mechanism of recovery from inactivation is less clear. After opening, *Sh* channels have a refractory period during which they are unable to open to another stimulus. The rate of this recovery could be determined by the C-terminus of *Sh* products (12), and a second inactivated state whose stability is determined by the C terminal end. How the C terminal end regulates the rate of recovery is unclear. One possibility is that the C terminal end also acts at the "ball receptor" by stabilizing the "ball" in its receptor. Thus, the C terminal end (thought to be cytoplasmic) could also act at this internal site. One of the mutations of the heptad repeat region (Chapter 2B) , V2A3, although showing intermediate alterations between the V2 and A3 mutants for most other properties, was uniquely altered in its recovery from inactivation; the recovery of wild-type 29-4 channels is complete within

approximately 1s while V2A3 does not completely recover within approximately 10s (unpublished results). This suggests that the C terminal end may also act at a site within or near the heptad-repeat region.

Multimeric Association. The currents seen in native *Drosophila* tissues (7-11, 18) do not appear to be identical to any of those seen in expression systems when a single *Sh* product is expressed. The native currents differ slightly in their properties of inactivation and recovery from inactivation. Furthermore, antibody and mRNA tissue-*in situ* hybridization studies show that different splice products are sometimes expressed in the same tissues (23, 24). This raises the question as to whether different *Sh* splice products can associate to form functional channels in the same cell. If so, what properties will these hybrid channels have? Functionally hybrid channels would provide a means for even greater physiological diversity of *Sh* channels.

As mentioned previously, all of the splice products of *Drosophila Sh* have similar voltage-dependences of activation and inactivation, K+ selectivity, and sensitivity to pharmacological agents (12, 25). In the study presented in Chapter 3 (26), a mutant (29-4(V2)) with a large shift in its voltage-dependence was co-expressed with other wild type *Sh* products which differed in either the N or C termini. It was shown that the hybrid channels form with high efficiency and that the rates of inactivation and recovery from inactivation are closer, but not equivalent to, the subunit type with the faster inactivation or recovery from inactivation. Thus, subtle or graded changes in the properties of *Sh* channels may be generated by the expression of different types of *Sh* products in the same cell. Other studies in *Drosophila* (27), as well as with different vertebrate *Sh* homologs (28, 29) also have shown that heteromultimeric channels form within the *Sh* class of K+ channel.

However, heteromultimers do not form between members of different classes of *Sh* family members (Chapter 3, refs. 26, 30). Thus, it appears that heteromultimerization provides a means for generating further diversity in a particular class of channel (*Sh, Shab, Shal* or *Shaw*), and that these classes form distinct functional groups.

The sites of subunit association and the process of assembly remains somewhat unclear for *Sh* family channels. The site of a *Drosophila Sh* mutation, Sh^{102}, which acts as an antimorph (heterozygous flies have less than half the normal *Sh* current amplitude (31)), has been identified. A single nucleotide substitution results in the generation of a stop codon in the H5 region (32). Thus, it has been suggested that the Sh^{102} product binds to wild-type subunits and inhibits their ability to form functional channels. Co-expression of wild type and Sh^{102} products, in *Xenopus* oocytes, results in a decrease in the amount of currents expressed by the wild type products; smaller C-terminal truncated products of *Sh*, up to the region between the S3 and S4 segments, produce similar reductions of *Sh* currents with no corresponding effects on co-expressed Na+ channel currents (Chapter 2B, ref. 6). This indicates that a region that is upstream of the S3-S4 region, which is different between *Sh* and other *Sh* family classes, appears to be responsible for a primary association in the assembly of channel subunits. In addition, large portions of the N termini are not necessary for association in *Sh* (27) and other *Sh* family members (33). This suggests that the primary assembly of *Sh* family products is determined within the S1-S3 region.

The decreases in current amplitude seen in the co-injections of wild-type and C-truncated products (Fig. 2 , Ch. 2B) would not be expected if the truncated products bound to wild type subunits as well as wild-type bound to itself. 1:1 co-injections of truncated and wild type products should give about

6% of the current; if functional *Sh* channels are tetrameric, and the truncated and wild-type products bound to each other equally well, there is only a 6.25% $(.50)^4$ chance that all of the subunits in a tetramer would be wild-type. In a 1:2 :: wild-type:Sh102 co-injection ratio only about 1% $(.333)^4$ of the wild-type currents would be expected to be expressed. This is clearly not the case (Fig. 2, Ch. 2B). Therefore, it is possible that*Sh* and Sh^{102} products may form dimers, however, only dimers with two wild-type products may form tetramers (Sh^{102} is truncated within a region thought to form an external portion of the pore-a site suggestive of intrasubunit contacts). Thus, *Sh* assembly could entail monomer-monomer and dimer-dimer formation with different sites important in determining the different types of association. In the 1:2 :: wild-type:Sh102 co-injections, about 11% $(.333)^2$ of the currents would be predicted to remain. This value is still somewhat smaller than those observed and therefore it may reflect the possibility that Sh^{102} products do not associate as well with wild-type subunits regardless of the level of association.

Activation. The mechanism of voltage-dependent activation, the most complex and interesting process in voltage-dependent ion channels, remains unclear. The kinetics of activation described by the Hodgkin and Huxley equations, although shown to be inaccurate in several parameters in Na+ channels (16, 19, 34, 35), continue to guide present experiments today that attempt to investigate the physical basis of voltage-dependent channel activation. The activation of voltage-dependent Na+ and K+ channels were suggested by Hodgkin and Huxley to depend on 3 and 4 independent charged gating particles, respectively. A similar model for the activation of *Sh* channels has recently been proposed with the addition of single channel analysis for some parameters.

According to this model (Fig. 1), the voltage-dependence of *Sh* channels is determined by 4 independent voltage-dependent rates (similar to the H&H model for K+ channels in squid giant axon), which are responsible for 4 independent gating charge movements each corresponding to voltage-sensors in each of the 4 subunits of a tetramer (18). The main differences between these models is that i) *Sh* produces an inactivating K+ current (in most cases, see above) and therefore the inactivation process must be included in models of *Sh* kinetics and ii) the addition of an extra closed transition in the activation process that is suggested to be a voltage-independent step.

This last transition was inferred from the single-channel flicker rate of channels in the open state; it was assumed that the flickering of open channels was due to transitions between the last closed state and the open state rather than between open and inactivated states. However, it is stated that

" It is possible that burst closings occur by a process that is independent of the opening pathway. "

i.e., through inactivation or block by non-permeant ions. Since the flicker rate or burst closing times appeared to be voltage-independent, it was suggested that the last closed to open state transition must be voltage-independent as well. Similar voltage-independent steps had been suggested previously in Na+ channel gating models to account for the time differences between gating charge transitions and the onset of ionic currents (34, 35).

Other models of Na+ channel activation showed that cooperative rate constants (of gating particle transitions) give qualitatively similar descriptions in voltage-dependence of both macroscopic and single-channel ionic currents (36). This suggests that a different gating mechanism is tenable, i.e.,

cooperativity between the gating charge transitions. Other types of channels, e.g., gap-juncton (37), Ach (38) and cGMP-gated (39-41) channels, are thought to undergo cooperative transitions.

Nature of the Voltage-Sensing Particles

Before the voltage-dependent Na+ channel had been cloned, Armstrong proposed a structural model for the voltage-sensor and the mechanism of voltage-dependent activation (19). In this model, the voltage-sensor is composed of salt-bridged basic and acidic transmembrane residues which are located in separate domains. Upon depolarization of the membrane, the two domains are displaced relative to one another; to account for the positive character of the gating currents the basic residues move outward and/or the anionic residues inward.

Once isolated, the S4 domain was an interesting characteristic feature found in each of the homology domains of the Na+ channel. In several models (42-45), this motif was suggested to be a transmembrane segment corresponding to the Armstrong voltage-sensor. The popular model for the mechanism of voltage-sensing mediated by the S4 (45) is as follows: 1) basic residues in this domain are thought to interact with and be stabilized within the membrane by acidic amino acids in other transmembrane segments (although there does not appear to be enough acidic residues in other transmembrane segments), and 2) changes in membrane potential sensed by the charged residues in this domain result in its translocation through the membrane in a sliding helical motion. (The mechanism by which this motion is translated into opening and closing of the channel pore has not been addressed). That the S4 domain acts as the voltage-sensor in this fashion

became popular as similar S4 motifs were discovered in virtually all of the voltage-dependent channel genes (13, 14, 46-57). In addition, chemical modification of basic and acidic functional groups could alter the voltage-dependence of ionic and gating currents (see ref. 34 for review).

Evidence for the involvement of S4 in the voltage-sensing apparatus of voltage-dependent ion channels awaited the use of site-directed mutagenesis and heterologous expression systems. In the rat II Na+ channel, the basic residues in the S4 domain were substituted for uncharged or acidic residues (58) and the corresponding ionic-current properties were examined in *Xenopus* oocytes. Substitution of basic residues was shown to alter the voltage-dependence of activation and inactivation, although sometimes in different directions (even though it has been shown that there is a strong coupling between them (16-19)). Furthermore, from estimates of the amount of gating charge from conductance-voltage curves (the slope of the conductance-voltage curve is thought to be determined by the number of gating charges, see ref. 59), a strong correlation was noted between the number of charged residues substituted in the S4 of homology domain I and the amount of estimated gating charge. This did not apply to charged residues in the second homology domain, which was studied less extensively. The actual gating charge properties have not been reported for any of these mutants. Finally, some of the mutations shifted the conductance-voltage curve in the hyperpolarizing direction while others shifted it in the depolarizing direction. This effect was suggested to depend on the location of the substituted residue with respect to the membrane and the electric field. Thus, all of the effects were proposed to be explainable strictly in terms of the electrostatic effects on charged voltage-sensing residues, and it was suggested that the S4 charged residues contained properties expected for the voltage-sensing particles.

More recent studies in *Sh* K+ channels have suggested that there are other important parameters that determine activation gating. *Sh* channels are more amenable to mutagenic analysis since they can consist of identical subunits (Na+ channels consist of homology domains which contain significant sequence differences, e.g., S4 domains have anywhere from 4-8 charges while in *Sh* all S4 domains presumably contain 7 (50, 51, 60-63)). In a study of *Sh*, the S4 domain basic residues (both Arg and Lys are within the *Sh* S4 motif) were independently substituted for 1) glutamine, or 2) Arg residues were replaced by Lys residues and vice versa (64). The slopes of the conductance-voltage and prepulse inactivation curves were altered in a manner inconsistent with the number of S4 gating charges substituted (since inactivation is apparently voltage-independent the voltage-dependence of inactivation has been suggested to be an indicator of the voltage-dependence of activation and thus its slope can also be used to estimate the number of gating charges, see refs. 18, 105). In the neutralization experiments (glutamine substitutions), based purely on electrostatic considerations, each mutation should decrease the slope of activation and inactivation curves (gating charge) by 1/7 or about 14%. However, only one of these mutations is reported to affect these slopes, and furthermore, it decreases the slope of the prepulse inactivation curve by about 250%. This was the largest observed effect on slope in this study and interestingly this particular residue is seven residues upstream of a leucine-heptad repeat (Chapters 2A , 2B, refs. 6, 65). Thus, the the observed effects for this mutation may be related to its position with respect to a helical heptad-repeat. The only other observed slope decrease (no substantial slope increases were reported) was for one of the Arg-->Lys mutations (the third residue in the leucine-heptad repeat, see Ch. 2B, Fig 1). Thus, the neutralization experiments did not produce uniform alterations in

estimated gating charge (slope), and a substitution that did not change charge also changed the estimated gating charge; these results were not predicted by electrostatic models. The actual gating charge properties of these mutant channels have not been reported. In addition, shifts along the voltage-axis were not uniform and therefore not interpretable in terms of interactions between the electric field and the S4 charged residues.

Thus, although the S4 domain appears to be involved in voltage-dependent gating, the nature of the charges involved in voltage-sensing remains unclear. It is possible that these charges represent dipoles of a combination of transmembrane segments in a given subunit or that they represent ions that interact with the channel in conformational transitions of activation. Furthermore, it appears that protein-protein interactions and the stabilities of specific conformational states are important in determining the conformational transitions involved in voltage-dependent activation, as might have been anticipated.

Transitions Independent of Gating Charge Influence Voltage-Dependence

In Chapter 2A (65), a mechanism for the transduction of gating charge movement into channel opening is suggested. This mechanism is based on subunit interactions through a conserved leucine-heptad repeat region that overlaps the S4 domain. It was suggested that the conformational transitions involved in voltage-dependent gating, and leading to channel opening, could be transmitted between subunits through the leucines in the heptad repeat, i.e., a cooperative process.

The conserved heptad periodicity suggests that this region contains a high helical content; a mutagenic characterization of the leucines in the heptad-repeat of *Sh* K+ channels, through conservative leucine--> valine substitutions, is presented in Chapter 2B. Some of these substitutions result in larger shifts in voltage-dependence and decreases in slope than any of the substitutions of the S4 charged residues (64). It appears that similar processes occur in Na+ channels since substitution of the first leucine residue in the heptad repeat (it is also in the S4 domain) of the second homology domain of the rat IIa Na+ channel shifts the voltage-dependence of activation by approximately +25 mV (66). Since the heptad mutants are conservative substitutions of uncharged residues it is difficult to explain their effects in terms of electrostatic effects on the S4 domain or in the context of earlier models. The most tenable interpretation in the context of the sliding-helix model (45) would be that steric obstructions could be introduced to S4, and affect gating charge movement through the heptad substitutions. Thus, alterations in gating charge movements could account for these effects.

Gating currents in the V1 and V2 mutants are currently being examined (67). It appears that the alterations in the ionic current properties of V1 and V2 (both the slope and the voltage-shift) do not have corresponding alterations in gating current properties; the major components of V1, V2 and wild-type gating currents appear to have identical voltage-dependent properties (Schoppa and Sigworth, unpublished results). Thus, if the heptad leucines are important in determining the transduction of charge movement into channel opening through some cooperative process it is not similar to the cooperative gating processes, modeled by Armstrong for the gating particles (36). Furthermore, there is some other voltage-dependent process, independent of the major component of gating charge movement, in V1 and

V2 channels. Most models suggest that the voltage-dependent properties of these channels are the direct result of the major component of gating charge transitions. Thus, it is not possible to explain the voltage-shifts and gating charge alterations of the V1 and V2 mutants in terms of these models; they must be altered or special exceptions as to the nature of V1 And V2 gating must be made. However, the effects of these mutants are consistent with the idea that the heptad-repeat region influences the transduction of charge movement into channel opening; possibly through α-helical hydrophobic interactions between subunits, as previously suggested (Chapter 2A, ref. 65).

If voltage-dependent, but gating-charge independent, transitions (C_5 - 0) are important in the gating of V1 channels, it should be asked whether this process is significant in wild type gating as well. Single channel analysis of *Sh* currents (18) have suggested that the last closed-open transition (C_5 - 0) is voltage-independent between -20 and +50 mV and thus such a transition does not appear to have a large effect on the activation of this step in wild type channels. However, the analysis was dependent on the assumption that the flickering of open channels is due solely to open-close transitions (see above).

In wild type channels, it is possible that potentials that are sufficient to move the gating charges at low voltages are also large enough for saturation of a voltage-dependent (C_5 - 0) transition, which is independent of gating-charge. Evidence that such a transition in wild type channels is not completely saturated might be provided by the observation that some mutations in the heptad-repeat region (e.g., A3, V4, see Ch 2B) are shifted in the hyperpolarizing direction, and have slightly steeper slopes. It appears unlikely that these conservative hydrophobic mutations affect gating charge transitions. Thus, there may be a C_5 - 0 which is faster in A3 and V4 channels in comparison to wild type channels. If so, voltage-dependent gating is at least

partially determined by the rate of a transition, independent of gating charge, in wild type channels. Furthermore, alterations in this transition (rather than in gating-charge transitions) could be utilized by channels that vary in their voltage-dependences. In addition, modulation of channel activity, e.g., through phosphorylation, may primarily alter this transition as well. Investigation of the gating currents of V4 and the mechanism of the voltage-dependent transitions, altered in V1 (C_5 - 0), are currently underway.

The recent flood of structure-function studies of *Sh* family K+ channels has revealed significant characteristics of these channels: external and internal regions of the pore, the inactivation ball and chain and several pharmacologically important regions. The structure-function relationships of the voltage-dependent gating mechanism, however, remains unclear. It appears that charged residues in the S4 domain are determinants of voltage-dependence, but hydrophobic residues also appear to play a significant role. The nature of the voltage-sensor and its relationship to gating charge has not been established. Furthermore, the voltage-dependence of these channels appear to be at least partially determined by transitions independent of the major component of gating charge. Thus, the processes that determine activation gating in voltage-dependent ion channels will continue to be of paramount importance in the forseeable future.

References

1. MacKinnon, R., Heginbotham, L. & Abramson T. "Mapping the receptor site for charybdotoxin, a pore-blocking potassium channel inhibitor." *Neuron* **5**, 767 (1990)

2. Guy, H.R. & Conti, F. "Pursuing the structure and function of voltage-gated channels." *TINS* **13**, 201 (1990)

3. MacKinnon, R. & Miller C. "Mutant potassium channels with altered binding of charybdotoxin, a pore-blocking peptide inhibitor." *Science* **245**, 1382 (1989)

4. MacKinnon, R. & Yellen, G. "Mutations affecting blockade and ion permeation in voltage-activated potassium channels. "*Science* **250**, 276 (1990)

5. Noda, M., Suzuki, H., Numa, S. & Stuhmer, W. "A single point mutation confers tetrodotoxin and saxitoxin insensitivity on the sodium channel II." *FEBS Lett.* **259**, 213 (1989)

6. McCormack, K., Tanouye, M.A., Iverson, L.E., Lin, J.W., Ramaswami, M., McCormack, T., Campanelli, J.T. Mathew, M.K. & Rudy, B."A role for hydrophobic residues in the voltage-dependent gating of *Shaker* K+ channels." *Proc. natn. Acad. Sci. USA* , in press

7. Salkoff, L.B. & Wyman, R. "Genetic modification of potassium channels in Drosophila *Shaker* mutants."*Nature* **293**, 228 (1981)

8. Wu, C.F. & Haugland F.N. "Voltage-clamp analysis of membrane currents in larval muscle fibers of Drosophila: alteration of potassium currents in *Shaker* mutants." *J. Neurosci.* **7**, 1307 (1985)

9. Salkoff, L. & Wyman, R. "Outward currents in developing Drosophila flight muscle." *Science* **212**, 461 (1981)

10. Solc, K.C., Zagotta, W.N. & Aldrich, R.W. "Single-channel and genetic analyses reveal two distinct A-type potassium channels in Drosophila." *Science* **236**, 1094 (1987)

11. Zagotta, W.N., Hoshi, T. & Aldrich, R.W. "Gating of single *Shaker* K+ channels in Drosophila muscle and in Xenopuys oocytes injected with *Shaker* mRNA." *Proc. natn. Acad. Sci. USA* **86**, 7243 (1989)

12. Iverson, L.E. & Rudy, B. "The role of divergent amino and carboxyl domains on the inactivation properties of potassium channels is derived from the *Shaker* gene of Drosophila." *J. Neurosci.* **10**, 2903-2916 (1990)

13. Stuhmer, W., Ruppersburg, J.P., Schroter, K.H., Sakmann, B., Giese, K.P., Perschke, A., Baumann, A. & Pongs, O. "Molecular basis of functional diversity of voltage-gated potassium channels in mammalian brain." *EMBO J.* **8**, 3235 (1989)

14. Ramaswami, M., Gautam, M., Kamb A., Rudy, B. Tanouye & Mathew, M.K. "Isolation and characterization of a family of human *Shaker* potassium channels." *Mol. Cell. Neurosci.*, in press

15. Connor, J. & Stevens, C. "Voltage clamp studies of a transient outward membrane current in gastropod neural somata." *J. Physiol.* **213**, 21 (1971)

16. Armstrong, C.M. & Benzinilla F. "Inactivation of the sodium channel. II. gating current experiments." *J. Gen. Phys.* **70**, 567 (1977)

17. Aldrich, R.W., Corey, D.P. & Stevens, C. "A reinterpetation of mammalian sodium channel gating based on single channel recording." *Nature* **306**, 436 (1983)

18. Zagotta, W.N. & Aldrich, R.W. "Voltage dependent gating of *Shaker* A-type potassium channels in Drosophila muscle." *J. Gen Physiol.* **95**, 29 (1990)

19. Armstrong, C.M. "Sodium channels and gating currents." *Physiol. Rev.* **61**, 644 (1981)

20. Hoshi, T., Zagotta, W.N. & Aldrich, R.W. "Biophysical and molecular mechanisms of Shaker potassium channel inactivation." *Science*, **250**, 533 (1990)

21. Armstrong, C.M., Benzinilla, F. & Rojas, R. "Destruction of sodium conductance inactivation in squid axons perfused with pronase." *J. Gen. Physiol.* **62**, 375 (1973)

22. Zagotta, W.N., Hoshi, T. & Aldrich, R.W. "Restoration of inactivation in mutants of *Shaker* potassium channels by a peptide derived from *Sh* B." *Science* **250**, 568 (1990)

23. Schwarz, T.L., Papazian,D.M., Caretto, R.C., Jan Y.N. and Jan L.Y. "Immunological characterization of K+ channel components from the *Shaker* locus and differential distribution of splicing variants in Drosophila." *Neuron* **2**, 119 (1990)

24. Tseng-Crank, J., Pollock, J. Hayashi, I. & Tanouye, M.A. "Expression of ion channel genes in *Drosophila*." *J. Neurogenet.* , in press

25. Timpe, L.C., Jan, Y.N. & Jan, L.Y. "Four cDNA clones from the Shaker locus of Drosophila induce kinetically distinct A-type potassium currents in Xenopus oocytes." *Neuron* **1**, 659 (1988)

26. McCormack, K., Lin, J.W., Iverson, L.E. & Rudy, B. "*Shaker* K+ channel subunits form heteromultimeric channels with novel functional properties." *Biochem. Biophys. Res. Comm.* **171**, 1361 (1990)

27. Isacoff, E., Jan Y.N. & Jan, L.Y. "Evidence for the formation of heteromultimeric potassium channels in Xenopus oocytes." *Nature* **345**, 530 (1990)

28. Chrisite, M.J., North, R.A., Osborne, P.B., Douglass, J. & Adelman, J.P. "Heteropolymeric potassium channels expressed in Xenopus oocytes from cloned subunits." *Neuron* **4**, 405 (1990)

29. Ruppersburg, J.P., Schroter, K.H., Sakmann, B., Stocker, M., Sewing, S. & Pongs, O. "Heteromultimeric channels formed by rat brain potassium channel proteins." *Nature* **345**, 535 (1990)

30. Covarrubias, M., Wei, A., McKinnon, D. & Salkoff, L. "Hybrid K+ channels are not formed between four subfamilies of K+ channel genes." *Neurosci. Abstr.* #6.4 (1990)

31. Haugland, F.N. & Wu, C.F. "A voltage-clamp analysis of gene-dosage effects of the *Shaker* locus on larval muscle potassium currents in Drosophila." *J. Neurosci.* **10**, 1357 (1990)

32. Gisselman, G., Sewing, S., Madsen, B.W., Mallart, A., Anguat-Petit, D., Muller-Holtkamp, F., Ferrus, A. & Pongs, O. "The interference of truncated with normal potassium channel subunits leads to abnormal behavior in transgenic Drosophila melanogaster." *EMBO J.* **8**, 2359 (1989)

33. Van Dongen, A.M.J., Frech, G.C., Drewe, J.A., Joho, R.H. & Brown, A.M. "Alteration and restoration of K+ channel function by deletions of the N- and C- termini." *Neuron* **5**, 433 (1990)

34. French, R.J. & Horn, R. "Sodium channel gating: models, mimics and modifiers." *Ann. Rev. Biophys. Bioeng.* **12**, 319 (1983)

35. Meves, H. "Hodgkin-Huxley: thirty years after." *Curr. Topics Membr. Trans.* **22**, 279 (1984)

36. Armstrong, C.M. & Matteson, D.R. "Sequential models of sodium channel gating."*Curr. Top. Membr. Trans.* **22**, 331 (1984)

37. Unwin, N. "The structure of ion channels in membranes of excitable cells." *Neuron* **3**, 665 (1989)

38. Changeaux, J.P., Devillers-Thiery, A. & Chemalli, P. "Acetylcholine receptor: an allosteric protein." *Science* **225**, 1335 (1984)

39. Kaupp, U.B. "Primary structure and functional expression from complementary DNA of the rod photoreceptor cyclic GMP-gated channel." *Nature* **342**, 762 (1990)

40. Haynes, L.W., Kay, A.R. & Yau, K.W. "Single cyclic GMP-activated channel activity in excised patches of rod outer segment membrane." *Nature* **321**, 66 (1986)

41. Zimmerman, A.L. & Baylor, D.A. "Cyclic GMP-sensitive conductance of retinal rods consists of aqueous pores." *Nature* **321**, 70 (1986)

42. Greenblatt, R.E., Blatt, Y. & Montal, M. "The structure of the voltage-sensitive sodium channel.".*FEBS Lett.* **193**, 125 (1985)

43. Guy, H.R. & Seetharamulu "Molecular model of the action potential sodium channel." *Proc. natn. Acad. Sci. USA* **83**, 508 (1986)

44. Noda, M., Ikeda, T., Kayano, T., Suzuki, H., Takeshima, H., Kurasaki, M., Takahashi, H. & Numa, S. "Existence of distinct sodium chanel messenger RNAs in rat brain.", *Nature* **320**, 188 (1986)

45. Catterall, W.A. "Molecular properties of voltage-sensitive sodium channels." *Ann. Rev. Biochem.* **55**, 953 (1986)

46. Tanabe, T., et al., "Primary structure of the receptor for calcium channel blockers from skeletal muscle." *Nature* **328**, 313 (1987)

47 Kamb, A., Iverson, L.E., & Tanouye, M.A. "Molecular characterization of *Shaker*, a Drosophila gene that encodes a potassium channel." *Cell* **50**, 405 (1987)

48.Tempel, B., Papazian, D.M., Schwarz, T.L., Jan, Y.N. & Jan, L.Y. "Sequence of a probable potassium channel component encoded at the *Shaker* locus of Drosophila." *Science* **237**, 770 (1987)

49. Baumann A., Krah-Jentgens, H., Muller, R., Muller-Holtkamp, S., Seidel, R., Kecskemethy, N., Casal, I., Ferrus, A. & Pongs, O. "Molecular organization of the maternal effect region of the *Shaker* complex of Drosophila: characterization of an I_A channel transcript with homology to vertebrate Na+ channel." *EMBO J.* **6**, 3419 (1987)

50. Tempel, B., Jan, Y.N. & Jan L.Y. "Cloning of a probable potassium channel gene from mouse brain." *Nature* **332**, 837 (1988)

51. Baumann, A., Grupe, A., Ackermann, A. & Pongs, O. "The structure of the voltage-dependent potassium channel is highly conserved from Drosophila to vertebrate central nervous systems." *EMBO J.* **7**, 2457 (1988)

52. Chandy, K.G., Williams, C.B., Spencer, R.H., Aguilar, B.A, Ghanshani, S., Tempel, B.L. & Gutman, G.A. "A family of three mouse potassium channel genes with intronless coding regions." *Science* **247**, 973 (1990)

53. Wei, A., Covarrubias, M., Butler, A., Baker, K., Pak, M. & Salkoff, L. "K+ channel diversity is produced by an extended gene family conserved in Drosophila and mouse." *Science* **248**, 599 (1990)

54. Butler, A., Wei, A., Baker, K.& Salkoff, L. "A family of putative potassium channel genes in Drosophila." *Science* **243**, 943 (1989)

55. Frech, G., Van Dongen, A.M.J., Schuster, G., Brown, A.M. & Joho, R. "A novel potassium channel with delayed rectifier properties isolated from rat brain by expression cloning." *Nature* **340**, 642 (1989)

56. McCormack T., Vega-Saenz de Miera, E. & Rudy, B. "Molecular cloning of a member of a third class of Shaker-gamily K+ channel genes in mammals." *Proc. natn. Acad. Sci. USA* **87**, 5227 (1990)

57. Vega-Saenz de Miera, E., Sen, K., Serodio, P., McCormack, T. & Rudy, B. "Description of a new class of potassium channel genes." *Neurosci Abstr.* #6.2 (1990)

58. Stuhmer, W., Conti, F., Suzuki, H., Wang, X., Noda, M., Yahagi, N., Kubo, H. & Numa, S. "Structural parts involved in activation and inactivation of the sodium channel." *Nature* **339**, 597 (1989)

59. Hille, B. "Ionic Channels of Excitable Membranes." (Sinauer, Sunderland, MA). (1984)

60.Kamb, A., Tseng-Crank, J., & Tanouye, M.A. "Multiple products at the Drosophila *Shaker* gene may contribute to potassium channel diversity." *Neuron* **1**, 421 (1988)

61. Pongs, O., Kecskemethy, N., Mueller, R., Krah-Jentzens, H., Baumann, A., Kiltz, H.H., Canal, I., Llamazares, S. & Ferrus, A. "*Shaker* encodes a family of putative potassium proteins in the nervous system of Drosophila.", *EMBO J.* **7**, 1087 (1988)

62. Schwarz, T.L., Tempel, B.L., Papazian, D.M., Jan, Y.N. & Jan, L.Y. "Multiple potassium channel components are produced by alternative splicing at the *Shaker* locus in Drosophila.", *Nature* **331**, 137 (1988)

63. Pfaffinger, P., Furakawa, Y., Kubo, T., Zhao, B. & Kandel, E.R. "Molecular analysis of potassium channels in identified cells of Aplysia." *Neurosci. Abstr.* (1990)

64. Papazian, D.M., Timpe, L.C. Jan, Y.N. & Jan, L.Y. "Alteration of voltage-dependence of *Shaker* potassium channel by mutations in the S4 sequence." *Nature* **349**, 305 (1991)

65. McCormack, K., Campanelli, J.T., Ramaswami, M., Mathew, M.K. Tanouye, M.A. Ivrson, L.E. & Rudy , B. "Leucine zipper motif update.", *Nature* **340**, 103 (1989)

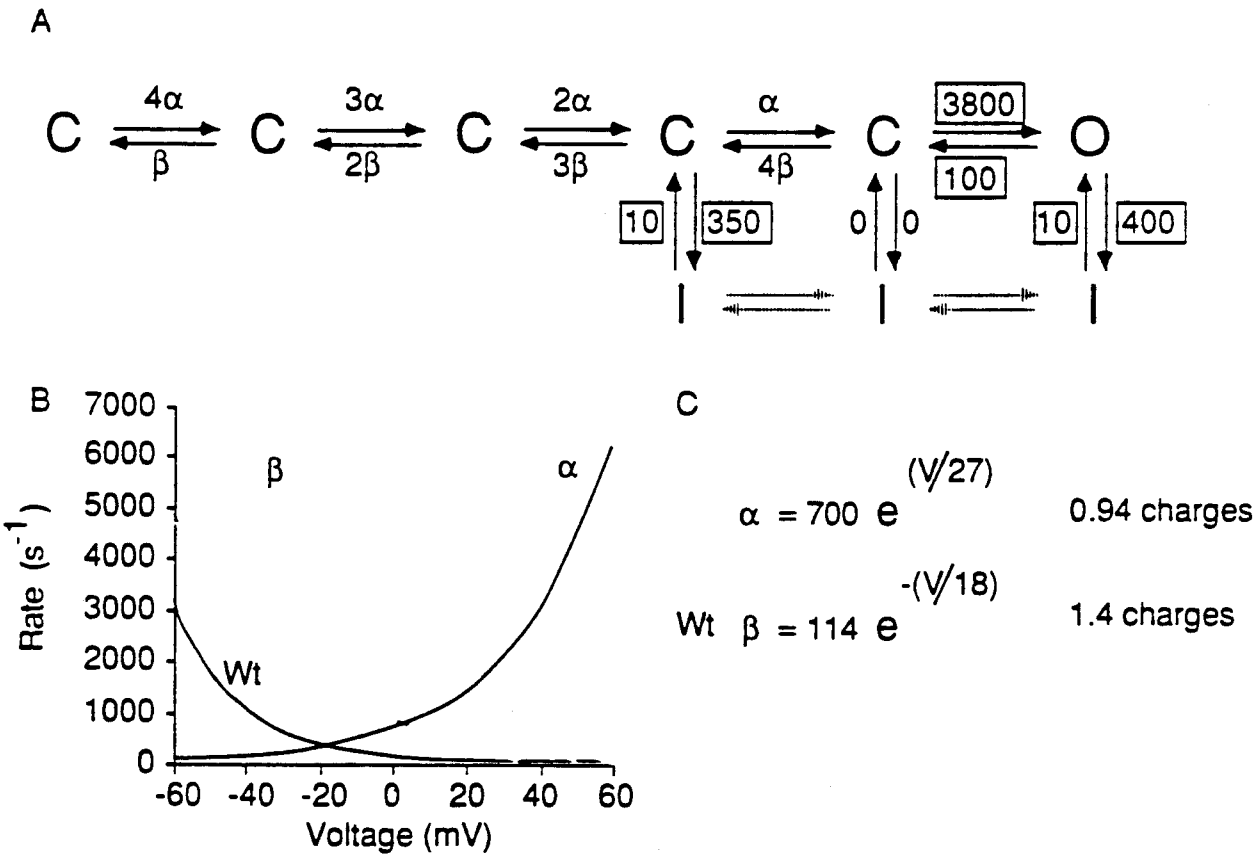
66. Auld, V.J., Goldin, A.L., Krafte, D.S., Catterall, W., Lester, H. A., Davidson, N. & Dunn, R. "A neutral amino acid change in segment IIS4 dramatically alters the gating properties of the voltage-dependent sodium channel." *Proc. Natn. Acad. Sci. U.S.A.* **87**, 323-327 (1990)

67. Schoppa, N., McCormack, K., Tanouye, M.A. & Sigworth, F.J. "High-time resolution recordings from oocytes injected with wild-type and V1 mutant 29-4 cDNAs." *Biophys J.* **59**, 196a (1991)

Figure 1 legend

A) State diagram of the model showing the absolute magnitudes of the voltage-independent rates (s^{-1}) and the relative magnitudes of the voltage-dependent rates. The rate constants that do not depend on voltage are boxed. Since this channel exhibited little voltage-independent closed state inactivation, the inactivation rate from the right-most closed state was zero. Some channels exhibited more blank sweeps at +50 mV, suggesting a slow inactivation rate from the right-most closed state. B) Exponential voltage dependence of the activation and deactivation rates. C) Equations for the activation and deactivation rates showing the equivalent charge movement associated with these rates.

Figure 1


A
C
4α
β
C
3α
2β
C
2α
3β
C
α
4β
C
3800
100
O
10
350
0
0
10
400
B
Rate (s-1)
7000
6000
5000
4000
3000
2000
1000
0
β
α
Wt
-60
-40
-20
0
20
40
60
Voltage (mV)
C
α = 700 e (V/27)
0.94 charges
Wt β = 114 e -(V/18)
1.4 charges

Appendix

Evidence for an Alternative Exon in the "Constant" Region of *Drosophila Shaker* K^+ Channels.

Ken McCormack[1] and Mark A. Tanouye[2]

[1] Ken McCormack, Division of Biology 216-76, California Institute of Technology, Pasadena CA 91125

[2] Mark A. Tanouye, Department of Entomology and Parasitology, University of California, Berkeley CA

Introduction:

The *Drosophila Shaker* (*Sh*) gene locus spans at least 120 kilobases of genomic DNA and contains at least 21 exons (1-3). *Sh* codes for several different voltage-dependent K^+ channels which are generated through alternative splicing. All of these products share a constant core region containing the first five transmembrane domains, however, several alternative exons exist for the amino and carboxyl ends. Physiological analysis of these products has shown that the divergent amino domains influence the kinetics of channel inactivation while the divergent carboxyl domains influence the rate of recovery from inactivation (4). Other properties of the channel, e.g., conductance, ionic selectivity, voltage-dependence, and sensitivity to 4-Aminopyridine (4-AP), tetraethylammonium (TEA) and charybdotoxin (CTX) appear to be governed by structures within the constant region of the *Sh* proteins (4, 5).

Here we provide evidence for the existence of an alternative exon in the "constant" region of *Sh*. This putative exon may be alternatively expressed as the last exon in the constant region which codes for the C-terminal half of the fifth transmembrane segment (S5) through the end of the H5 segment (3). Our results support the idea that this region may form part of the channel pore (6) since amino acid differences in this exon alter the sensitivity of the channel to block by TEA. Furthermore, it suggests that *Sh* may encode K^+ channels with different conductances. In addition, the expression of this exon in *Drosophila* muscle could account for the observed insensitivity of *Sh* channels in this tissue (7, 8) to block by CTX.

Materials and Methods:

For all Polymerase Chain Reaction (PCR) procedures 40 cycles of 55°C annealing, 72°C extension and 94°C melting temperatures were used. PCR generated fragments were cut with Eco R1 ends and were ligated into Eco R1 digested pIBI30 vector (IBI) and sequenced with the Sequenase (US Biochemicals) kit. The PCR oligonucleotides depicted in Figure 1 contained Eco R1 ends which are not shown.

Southern blots of *Drosophila* genomic DNA were used to determine optimal stringencies of hybridization (22°C, 50% formamide) and washing (55°C, .2x SSC) for the PCR generated sequences. ATP^{32}-labeled probes were generated by Klenow extension. Southern blots of phage DNA isolated from the *Sh* locus were hybridized and washed at identical stringencies.

The screening of cDNA libraries was done at the same stringencies as above. Isolated phage cDNA clones and the *Sh* phage genomic DNA were restriction mapped with Bam H1, BstE II, Eco R1, Eco RV, Hind II and Sal 1. PCR on phage genomic DNA was performed using a sense oligo to the novel exon sequence and an antisense oligo to the previously reported exon sequence.

Substitution of the putative exon amino acid residues were done on the 29-4 Sh cDNA using PCR. After PCR the altered 29-4 clone (29-4a) was sequenced to verify that only the appropriate amino acid changes were made. 29-4 and 29-4a RNA was prepared and injected into *Xenopus* oocytes using the same methods cited in Chapters 2 and 3.

Results:

Degenerate oligonucleotides of sequences which are conserved between *Drosophila*, mouse and rat *Shaker* channels and which code for the end of the fifth hydrophobic domain (S5), and the beginning of the H5 region, were used for PCR on *Drosophila* genomic DNA (Fig. 1). Identical degenerate oligonucleotides were used previously by Kamb, et al., (9) on human genomic DNA. In the *Drosophila Sh* gene, this region has been suggested to be part of a larger constant core region, common to all *Sh* products, which does not contain alternative exons (3). Sequence analysis of the PCR generated fragments revealed several copies of two conserved nucleotide and deduced amino acid sequences. One of the sequences corresponds to the 15th exon of *Sh* (1) located between splice sites 9 and 10, described in Schwarz et al. (3), while the other identified a novel sequence (Fig. 1).

Southern blots of restriction-digested *Drosophila* genomic DNA were used to determine the stringencies of hybridization for the PCR generated sequences; the labeled PCR probes identified different restriction-digested *Drosophila* genomic DNA fragments when hybridized at 22°C, in 50% Formamide and washed in .2x SSC at 55°C. To make sure that the labeled novel-sequence DNA would not cross-hybridize with higher concentrations of *Sh* exon 15 DNA southern blots of *Sh* cDNAs were performed. At the same stringency no detectable hybridization was observed between the labeled novel-sequence probes and the *Sh* cDNAs.

To determine whether the novel sequence might code for an alternative exon in the *Sh* locus or whether it coded for an analogous region in another K^+ channel gene, labeled probes corresponding to the novel

sequence were hybridized to phage genomic clones generated from the *Sh* locus (10). Interestingly, we detected hybridization to a clone known to contain *Sh* exon 15. PCR amplification of these phage genomic clones using a sense oligo to the novel sequence and an antisense oligo from exon 15 produced an approximate 1.3 kilobase (kb) fragment (data not shown). This indicates that the novel sequence is located 1.3 kb upstream of exon 15, and thus, the novel sequence might represent an alternative exon or a pseudo-exon in the *Sh* gene complex.

Using labeled novel-sequence probes we screened approximately 1 million phage cDNA clones from an adult head cDNA library, and isolated 24 independent positive clones. Sequence and restriction analysis revealed that all of the cDNAs isolated were incompletely processed *Sh* transcripts. Restriction mapping of some of these cDNAs revealed identical restriction patterns to those of a genomic fragment subcloned from the region encoding exon 15. Furthermore, sequence analysis revealed that exon 15 was contained in some of these cDNAs, and PCR amplification between the exon 15 and the novel sequence produces a 1.3 kb band in these cDNAs identical in size to those seen in amplifications of genomic DNA. Thus, these cDNAs sequences probably represent genomic DNA sequence from the *Sh* locus.

Sequence analysis of these cDNAs also revealed that, in fact, the novel-sequence DNA obtained from PCR was part of a larger region of homology with exon 15 (Fig. 2). We term that region here, exon 15a, since it is located 5' to exon 15 in the *Sh* locus. The conservation of the deduced amino acid sequence between exons 15 and 15a suggests that exon 15a may code for an alternative exon from the proposed "constant" region of *Sh*. Furthermore, exon 15a has similar consensus intron/ exon boundaries to that of exon 15 (Fig. 3).

In an attempt to determine whether exon 15a coded for a functional *Sh* product the deduced amino acid sequence of exon 15a was introduced into a 29-4 *Sh* cDNA (4). The 29-4 and 29-4a (29-4 with the exon 15a substitutions) cDNAs were used as a template for production of RNA. 29-4a and 29-4 RNAs were injected into *Xenopus* oocytes which were recorded from using a two-microelectrode voltage clamp (see Chapters 2 or 3). Both the 29-4 and 29-4a RNAs produced outward currents which were similar in the voltage-dependence of activation and inactivation, as well as in the rate of inactivation (data not shown). This suggests that exon 15a would produce functional channels if spliced into endogenous *Sh* transcripts containing either the 4 or 37 3' ends since the Thr --> Arg substitution would be identical when either end is a 3' splice partner. In addition, there were interesting pharmacologicak alterations in the currents produced by the amino acid substitutions of exon 15a: the sensitivity to TEA was substantially decreased in the 29-4a currents (Figure 4).

Discussion

Since we were unable to obtain full length clones containing exon 15a the possibility remains that it represents a pseudo-exon which is not expressed in *Sh* products. However, we think that this is an unlikely possibility for several reasons. First, it has been difficult to obtain full length *Sh* cDNA clones in general; the collection of full length *Sh* cDNAs which we have obtained have all come from a single adult head cDNA library (2). If exon 15a is expressed in *Sh* products in low quantities, or predominantly in other tissues or other developmental stages, it may be difficult to obtain these

clones. In addition, the sequence degeneracy of exon 15a in comparison to exon 15 is quite large in comparison to the degree of amino acid sequence conservation; of the 23 differences in nucleotide sequence only four result in translated amino acid differences (Fig. 2). A pseudo-exon in the human α-crystallin gene shows approximately the same relative number of nucleotide changes as a function of sequence length, but virtually every change in nucleotide sequence results in changes in the deduced amino acid sequence (11). Furthermore, there is a missense mutation in the α-crystallin pseudo-exon while no such alterations exist in exon 15a. A ratio of 4 amino acid substitutions per 23 changes in nucleotide sequence is difficult to explain except in terms of the conservation of amino acid sequence, and thus the functional expression of the exon. The fact that exon 15a has retained consensus splices sites (Fig. 3), and that the amino acid substitutions of exon 15a produced functional *Sh* currents (Table 1), also suggest that exon 15a is a bonafide *Sh* exon.

Mutagenic studies in the H5 region have produced Sh products with altered CTX (12) and TEA (6) sensitivity supporting the idea that H5 may form part of the channel pore. TEA is thought to block the open channel by physically obstructing the pore and thus inhibiting K^+ conductance (13). CTX appears to compete for channel block with TEA in Ca^{++} activated K^+ channels and therefore may also bind near an external portion of the *Sh* channel pore. Furthermore, after some mutations of the last residue coded for by exon 15 (Thr) and exon 15a (Arg), alterations in single channel conductance (more pronounced for inward currents) were observed (6). In fact, this was the only residue reported (T449) in the H5 region for which substitutions substantially altered TEA sensitivity and single channel conductance.

In Figure 4 we show that TEA sensitivity is altered in 29-4a currents. Since 29-4a and the previously reported Thr-->Arg mutation (6) have similar decreases in TEA sensitivity it is possible that the substitution of this residue alone is responsible of the alterations in TEA block of 29-4a currents. If so, it is likely that the 29-4a currents would also have the conductance changes reported for the Thr--> Arg mutation. Thus, the *Sh* locus may code for products with different conductances. However, it is also possible that the other amino acid substitutions in exon 15a products suppress the changes in single channel conductance from the the Thr--> Arg mutation or that they produce larger conductance alterations. Single channel studies are needed to resolve this issue.

In addition, mutations of this residue (T449) lower the affinity for CTX sensitivity more than three orders of magnitude (12). It has been shown that CTX does not block *Sh* currents expressed in *Drosophila* pupal muscle (7) or transformed myotubes (8) at concentrations of 50 nM. For *Sh* products expressed in Xenopus oocytes, however, channels are almost completely blocked by 20 nM CTX (13). It has been suggested that the differences in *Sh* CTX sensitivity in these preparations is due to differences in glycosylation or other posttranslational processing in the two preparations. However, exon 15a may be expressed in *Drosophila* muscle; in transformed myotubes heteromultimers of the different types of channel products may also be insensitive to CTX. Heteromultimers of other *Drosophila Sh* splice products have been shown to form functional channels (15, 16).

The *Drosophila Sh* gene has previously been shown to produce a diverse collection of K+ channel products through alternative splicing. However, the previously reported diversity of *Drosophila Sh* products was limited to splicing alterations in the 5' and 3' ends (1-3). These regions have

been shown to determine the rates of inactivation and recovery from inactivation, respectively (4). Thus the functional diversity of *Sh* products was primarily limited to alterations in these parameters: this diversity, however, may be quite complex since these alternatively spliced products can form heteromultimeric and functionally hybrid channels (15, 16). Here we report evidence for alternative splicing in the last exon in the "constant" region (exons 15 and 15a), suggesting that the physiological diversity of *Drosophila Sh* products is greater than previously reported.

It has been proposed that the regions not included in the alternatively spliced ends determine a "constant" region for *Drosophila Sh* products which extends from exon 7 to exon 15 (3). The putative exon 15a is arranged 5' to exon 15 placing it between exon 15 and exon 14. It is possible that there are other alternative exons which could code for exon 15 however it does not appear that they would be 5' to exon 15a since the sequencing of another 2.5 kb 5' to exon 15a revealed no other exons and we had not reached exon 14.

Exon maps of the *Sh* locus show that the size of intron regions becomes quite large after exon 14. This may indicate that the constant region of *Sh* is actually contained between exon 7 and exon 13 as previously suggested (1, 2). If this were the case there should be alternative exons which code for exon 14. This region codes for the S4 domain and a leucine heptad-repeat region which are important determinants in voltage-dependent gating, and possibly outward conductance (see Introduction). A recent report has described a *Sh* current in *Drosophila* ommatidia with a much more hyperpolarized voltage-dependence (17) than any previously reported for *Sh* . This is consistent with the idea that there may be alternative exons coding for this region of *Sh* products as well. Thus, the *Sh* gene may code for products which are functionally diverse in voltage-dependence and outward

conductance (exon 14), pharmacology and inward conductance conductance (exon 15), in addition to inactivation (5' ends) and recovery from inactivation (3' ends).

References

1. Pongs, O., et al., *EMBO J.* **7**, 1087 (1988)

2. Kamb, A., Tseng-Crank, J., & Tanouye, M.A. (1988) *Neuron* **1**, 421-430

3. Schwarz, T.L., et al., *Nature* **331**, 137 (1988)

4. Iverson, L.E. & Rudy, B. *J. Neurosci.* **10**, 2903-2916 (1990)

5. Timpe, L.C., et al., *Neuron* **1**, 659 (1988)

6. MacKinnon, R. & Yellen, G. *Science* **250**, 276 (1990)

7. Elkins, T., Ganetzky, B. & Wu, C.F. *Proc. natn. Acad. Sci. USA* **83**, 8415 (1986)

8. Zagotta, W.N., et al., *Neuron* 3, 773-82 (1989)

9. Kamb, A., et al., *Proc. natn. Acad. Sci. USA* 86, 4372 (1986)

10. Kamb, A., Iverson, L.E., & Tanouye, M.A. (1987) *Cell* **50**, 405-413

11. Jaworski, C.J. & Piatigorsky *Nature* **337**, 752 (1989)

12. MacKinnon, R., Heginbotham, L. & Abramson T. *Neuron* **5**, 767 (1990)

13. Hille, B. *Ionic Channels of Excitable Membranes*, (Sinauer, Sunderland, MA). (1984)

14. MacKinnon, R., Reinhart, P.H. & White, M.M. *Neuron* **1**, 997 (1988)

15. McCormack, K., Lin, J.W., Iverson, L.E. & Rudy, B. *Biochem. Biophys. Res. Comm.* **171**, 1361-71 (1990)

16. Isacoff, E., et al., *Nature* **345**, 530 (1990)

17. Hardy, R. *Gordon Conference Seminar* (1990)

18. Tempel, B., Jan Y.N. & Jan, L.Y. *Nature* **332**, 837 (1988)

Figure Legends

Figure 1. A) Degenerate oligonucleotides (K-2 and K-5) and deduced amino acids of sequences between segments S5 and H5 of mouse (MBK1, see ref. 18) and *Drosophila* (Sh, see ref. 2) *Shaker* K+ channels. Residues which are conserved between mouse and *Drosophila* are indicated by stars. B) Deduced amino acid sequences for the PCR generated fragments. Dashes indicate identical amino acids while the underlined residues indicate the sites of amino acid differences. The top sequence corresponds to that reported for exon 15 (1, 3) while the bottom sequence has not been previously reported.

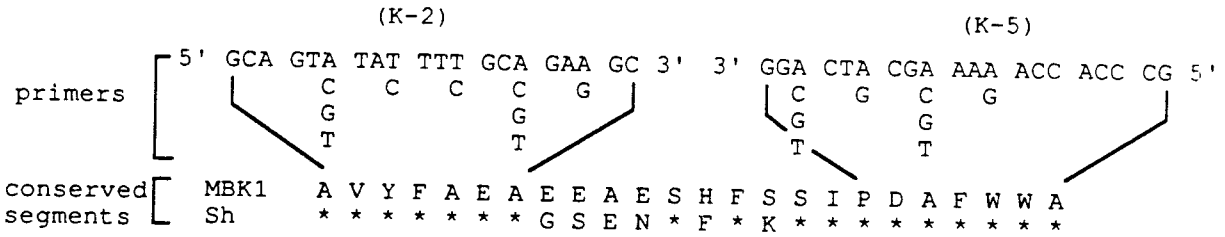
Figure 2. Nucleotide sequences of *Sh* exon 15 and a putative alternative exon 15a. Differences in nucleotide sequence are underlined where they do not result in changes in amino acid composition. Differences in nucleotide sequence which do result in amino acid composition are in bold face print. All of the deduced amino acid differences are indicated. The last nucleotide of the last codon in these exons is determined by alternatively spliced 3' exons. Two have been identified thus far, the type 4 3' end, which would place an adenosine in the last codon, and the type 37 3' end which would place a guanosine in the last codon (2, 3). Both of these codon insertions would result in the translation of an arginine residue at this position. Although none have been reported thus far another 3' end splice donor beginning in C or T would result in the translation of a Ser at this position.

Figure 3. The potential 5' and 3' splice sites for exons 15 and 15a are shown along with eukaryotic consensus splice sites. Potential intron/exon boundaries for exons 15 and 15a are analogous. Potential noncoding regions are indicated in lower case letters while potential coding regions are indicated in upper case letters. Nucleotide changes in exon 15a which do not result in changes in amino acid sequence are underlined and those that do result in amino acid changes are in bold.

Figure 4. The magnitude of ionic currents produced at +30 mV test potentials, from a holding potential of -90 mV, in the presence (I) or absence (Io) of the indicated concentrations of TEA.

Figure 1

A



B

Sh sequence	GSENSFFKSI
novel sequence	--DS------

Figure 2

```
 EXON 15    :.GC GTC GTA CTC TTC TCA TCG GCG GTT TAT TTT GCG
 EXON 15a   :.GC GTG GTC CTG TTC TCG TCG GCG GTT TAT TTC GCG


                 Glu Asn                                 Ala
GAA GCT GGA AGC GAA AAT TCC TTC TTC AAG TCC ATA CCC GAT GCA
GAG GCT GGC AGC GAC AGT TCC TTT TTC AAG TCG ATA CCG GAC GGC
                 Asp Ser                                 Gly


TTT TGG TGG GCG GTC GTT ACC ATG ACC ACC GTT GGA TAT GGT GAT
TTC TGG TGG GCT GTG GTG ACC ATG ACG ACG GTG GGA TAT GGT GAT

    Thr
ATG AC-->A in H4, G in H37 3'ends
ATG AG-->"                      "
    Arg/
```

Figure 3

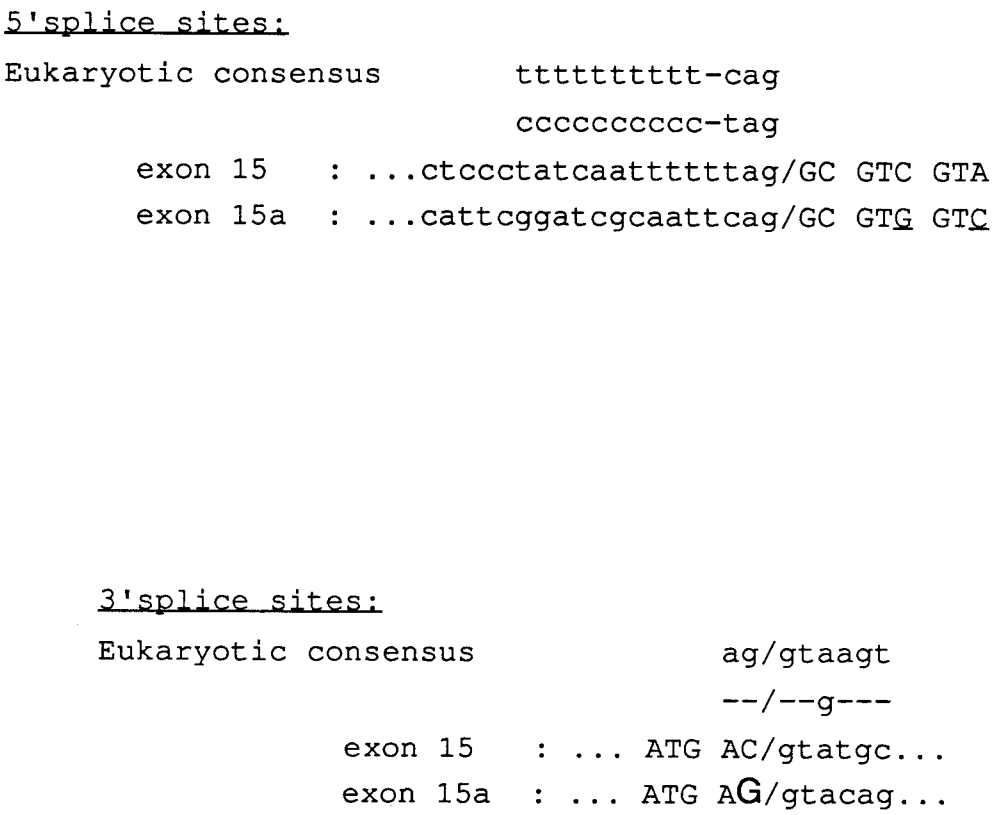
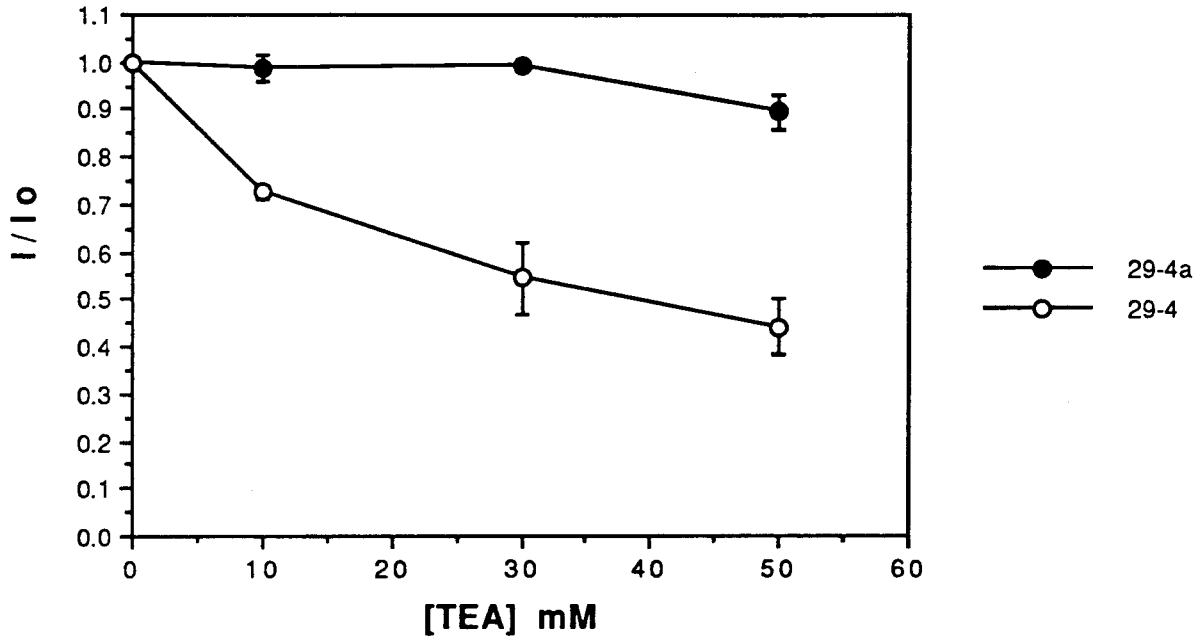

5'splice sites:
Eukaryotic consensus
ttttttttttt-cag
ccccccccccc-tag
exon 15 : ...ctccctatcaatttttttag/GC GTC GTA
exon 15a : ...cattcggatcgcaattcag/GC GTG GTC
3'splice sites:
Eukaryotic consensus
ag/gtaagt
--/--g---
exon 15 : ... ATG AC/gtatgc...
exon 15a : ... ATG AG/gtacag...

Figure 4


1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
I / Io
0
10
20
30
40
50
60
[TEA] mM
29-4a
29-4