MOLECULAR GENETIC STUDIES ON VOLTAGE-GATED ION CHANNELS

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Mani Ramaswami

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ABSTRACT

Several different methods have been employed in the study of voltage-gated ion channels. Electrophysiological studies on excitable cells in vertebrates and molluscs have shown that many different voltage-gated potassium (K^+) channels and sodium channels may coexist in the same organism. Parallel genetic studies in *Drosophila* have identified mutations in several genes that alter the properties of specific subsets of physiologically identified ion channels. Chapter 2 describes molecular studies that identify two *Drosophila* homologs of vertebrate sodium-channel genes. Mutations in one of these *Drosophila* sodium-channel genes are shown to be responsible for the temperature-dependent paralysis of a behavioural mutant *para* ^{ts}. Evolutionary arguments, based on the partial sequences of the two *Drosophila* genes, suggest that subfamilies of voltage-gated sodium channels in vertebrates remain to be identified.

In *Drosophila,* diverse voltage-gated K^+ channels arise from alternatively spliced mRNAs generated at the *Shaker* locus. Chapter 3 and the Appendices describe the isolation and characterization of several human K^+-channel genes, similar in sequence to *Shaker*. Each of these human genes has a highly conserved homolog in rodents; thus, this K^+-channel gene family probably diversified prior to the mammalian radiation. Functional K^+ channels encoded by these genes have been expressed in *Xenopus* oocytes and their properties have been analyzed by electrophysiological methods. These studies demonstrate that both transient and noninactivating voltage-gated K^+ channels may be encoded by mammalian genes closely related to *Shaker*. In addition, results presented in Appendix 3 clearly demonstrate that independent gene products from two K^+-channel genes may efficiently co-assemble into heterooligomeric K^+ channels with properties distinct from either homomultimeric channel. This finding suggests yet another molecular mechanism for the generation of K^+-channel diversity.

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Chapter 1

Introduction

Electrical Activity in Biological Membrane: A Brief History

Luigi Galvani discovered in 1791 that current applied to the sciatic nerve of a frog caused reproducible contraction of the innervated muscle. From this experiment he hypothesised that "animal electricity" was involved in nerve activity. In about 1850, Matteucci and Du Bois-Reymond first recorded electrical currents in animal tissue, in injured muscle fiber, using newly developed galvanometers. The surface of the muscle fiber was electropositive compared to the injured site; current flowed in the external circuit from the electropositive surface to the injured site. This current diminished during activity. Thus the active site on the muscle fiber became negative relative to the resting surface. Julius Bernstein observed that this electronegativity propagated like a wave down the fiber, with a velocity approximately equal to that determined by Helmholtz for the rate of propagation of the nerve impulse. Since then, nerve impulses have been associated with propagating waves of membrane depolarization (1).

It was apparent to the 19th century physiologists, that electric currents in neurons were probably carried by ions. Several ideas and notions about membrane potentials, most significantly those of Walter Nernst, were synthesised in the "membrane theory" by Bernstein in 1902 . The rationale for this theory is given below.

The ionic concentrations in the cell interior vary substantially from the extracellular space. The various concentration gradients and membrane ionic permeabilities cause electrical potentials across neuronal membranes. This potential for a single ion "I" may be defined by the Nernst equation as:

$$E_I = RT/nF \ \ln\{[I]_{out}/[I]_{in}\}.$$

In the above equation the Nernst potential "E" is given in Volts when "R" (the universal gas constant) is in Joules/ mole-K, "T" (the ambient temperature) is in K, "n" is the valence of the ion, F (Faradays constant) is in coulombs/mole, and $[I]_{out}$ and $[I]_{in}$ represent extracellular and intracellular concentrations of the ion I.

In biological membranes, where several ionic species must be considered, the transmembrane potential is given by the equation:

$$V_m = \Sigma(g_I \times E_I)/ \Sigma g_I$$

Here "Σ" represents the sum over all ionic species "I," and g_I represents the membrane conductance to the ion "I."

Thus, the membrane potential is dependent on both the Nernst potentials of the individual ions and on the conductance of the membrane to specific ions. A list of the typical intracellular and extracellular concentrations of different ionic species is shown in Table 1. Also shown are the computed values of Nernst equilibrium potentials for each ion. It was known in the 19th century, that K^+ is at a higher concentration inside the cell than outside. The membrane theory proposed that at rest the excitable membrane is selectively permeable to potassium ions. Thus, the resting potential of the cell is negative, close to the potassium equilibrium potential. Bernstein went on to propose that neuronal stimulation causes a large local increase in membrane permeability to all ions, in a process now referred to as "membrane breakdown." The resultant membrane depolarization stimulates adjacent points of the nerve cell where the same process occurs. Thus a wave of depolarization propagates down the length of the axon.

The conclusions above, on the nature of excitable membrane, were based on a large amount of plausible, circumstantial evidence. Crucial technical advances, such as the use of cathode ray oscilloscopes for electrophysiological measurements by Erlanger and Gasser, and the invention of the voltage clamp apparatus by Kenneth Cole and Howard Curtis, allowed more accurate measurements of membrane potentials. Equally crucial was the use of the squid giant axon: it allowed intracellular recordings to be made for the first time, and also, the chemical analysis of axoplasm to accurately determine intracellular ionic concentrations. These advances set the stage for decisive experiments conducted between 1937 and 1952 by a small group of researchers including Alan Hodgkin, Andrew Huxley and Bernard Katz in Great Britain, and Curtis Cole and Howard Curtis in the United

States. These experiments transformed the main features of the membrane theory, from plausible hypothesis, to estabilished fact. It became clear that nervous impulses are propagated as electrical signals; that action potentials and synaptic potentials result from changes in membrane permeability to specific ions. The exact mechanisms by which membrane permeability is regulated remained unknown, until much later. These seminal papers have been compiled into a single volume by Cooke and Lipkin (1972) and are briefly reviewed below (2).

A major departure from the membrane theory stemmed from the discovery that, during an action potential, membrane potential not only ceased to be negative but actually reversed in sign and became positive. This positive "overshoot" was explained by the sodium theory of Alan Hodgkin and Bernard Katz (3). Hodgkin, Katz and Andrew Huxley clearly demonstrated that during the action potential, the membrane became briefly selectively permeable to sodium ions and the membrane potential approached the sodium equilibrium potential. Hodgkin and Huxley showed that the permeability to sodium decayed with time in a process they called inactivation. Both the inactivation of sodium conductance and activation of a potassium conductance forced the membrane potential back to the resting value. In the now famous Hodgkin and Huxley model, they suggested that sodium and potassium permeabilities behave independently; that independent membrane-bound particles, sensitive to transmembrane potential, behave as gates to control sodium and potassium permeabilities. Although proposed from studies on the squid giant axon, this model satisfactorily explains nerve membrane activity in a variety of organisms.

The mechanisms underlying synaptic transmission were roughly understood by the 1960s. As early as the 1840s, Claude Bernard showed that curare alkaloids (active ingredients of Amazonian arrow toxins) specifically blocked synaptic transmission at the frog neuromuscular junction without affecting either nervous or muscular excitability. Otto Loewi showed in the 1920s that excitation of the frog vagus nerve was coupled to the hyperpolarization of innervated muscle membrane via the secretion of acetylcholine

("*Vagusstoff* ") (4). It was later shown that acetylcholine, released by motor neurons innervating vertebrate striated muscle, caused depolarization of muscle membrane resulting in muscle contraction (5). Thus acetylcholine acts as a neurotransmitter secreted in response to electrical depolarization of the presynaptic nerve terminal to effect electrical changes in the post-synaptic membrane. Its effect may be inhibitory as in the frog vagus nerve or excitatory as in vertebrate neuromuscular junction. The underlying mechanisms linking depolarization of presynaptic membrane to neurotransmitter release were identified in classical experiments conducted largely by Bernard Katz and his coworkers (6, 7, 8, 9). A variety of neurotransmitters other than acetylcholine have since been identified in different synapses. This form of synaptic coupling involving a chemical neurotransmitter is most common; such synapses are called chemical synapses. A second rarer kind of synapse is the electrical synapse, where the cytoplasms of two cells are in direct physical and electrical contact. The region of membrane apposition between the cells is a specialized composite organelle known as a tight junction. Such synapses were first identified in crayfish motor neurons but have since been found in a variety of organisms (10).

Above, I have summarized the early knowledge of nervous system physiology gleaned by biophysicists in the era before molecular studies on nerve-membrane proteins. The next major advance in the study of membrane excitability was the elucidation of the nature of the mechanisms controlling specific ion permeabilities.

Ion Channel Proteins Determine Membrane Ionic Conductances

Membrane potential changes involve the passage of ions into out of cells. We now know that this occurs through membrane proteins called ion channels. While it was clear from Hodgkin and Huxley's experiments that specific mechanisms regulating the conductance of membranes to sodium and potassium ions are present in nerve membrane, the nature of these mechanisms was the subject of much speculation till the mid 1960s. In 1964, tetrodotoxin (TTX) a toxin from puffer fish (later identified in a variety of species including

salamanders and frogs) was shown to block the rise in sodium conductance in lobster giant axons without affecting potassium or leakage conductances (11). This specific effect on sodium conductance was observed in several preparations including frog myelinated axons and the squid giant axon. The same effects were observed with an independently isolated, structurally related, toxin called saxitoxin (STX). The quaternary ammonium ion, tetraethyl ammonium (TEA), was found to specifically block the rectifying potassium conductance in the squid giant axon (12). Thus the two ionic pathways were clearly separated by these pharmacological agents, in a manner similar to the specific inhibition of enzymes. It was generally accepted from these and other studies that independent ion channnels coexist in excitable membrane, a belief compatible with the assumptions made by Hodgkin and Huxley.

Other toxins were identified that bound sodium channels with different affinities in resting and depolarized membrane. The most reasonable interpretation of these results was that sodium channels undergo conformational changes on membrane depolarization exposing or masking toxin binding sites during the process (13, 14). If this were true, it followed that charged groups were present in the structure to sense membrane potential, and that these charges moved in reponse to membrane depolarization. Currents asociated with the movements of the hypothetical gating charges were recorded by Armstrong and Benzanilla (15). By the early 1970s, it became quite clear that ion channels are membrane proteins that undergo conformational changes that enable them to act as ion-selective pores in the membrane. The change in membrane permeability observed in skeletal muscle membrane upon the application of acetylcholine is mediated by ion channels that "open" in response to acetylcholine binding. The increase in sodium and potassium permeabilities observed by Hodgkin and Huxley in squid axon are mediated by ion channels that open in response to membrane depolarization. The immense diversity of distinct channel types in even simple nervous systems was however not appreciated till much later.

Shortly after Hodgkin and Huxley's pioneering work, microelectrodes began to be used widely for intracellular recordings of action potentials in nerve and muscle from several biological phyla. A wide variety of action potential waveforms and firing patterns were observed. It was believed early on that all electrically excitable cells had sodium and potassium conductances similar to the squid giant axon; that differences observed in various action potential waveforms occurred because of small, species-specific differences in time and voltage dependences of the kinetic parameters of the channels. However, several distinct types of ion channel, with varied kinetic properties, were soon found to be present in the same organism. It is now apparent that the squid giant axon has fewer kinds of channel than almost any other cell type that has been examined; it is clear that action potentials in most excitable cells involve varying contributions from a very large number of different channel types. The identification of all channels contributing to a complex action potential, for example that of a heart Purkinje cell, may be an extremely difficult process.

New channel types continue to be identified, as more excitable cells are analyzed with varied pharmacological agents and electrophysiological methods. The "patch clamp" technique, pioneered by Erwin Neher and Bert Sackmann in the late 1970s, has allowed the analysis of currents through a single channel molecule. Using this method it is possible to achieve gigaohm seals between an electrode and a small patch of biological membrane and thus measure 10^{-12} amperes of current (current through a "normal" channel is in the range between 5 and 50pA). The patch may be detached from the cell in an inside-out or outside-out conformation and examined in a variety of experimentally chosen conditions. This allows a description of channel kinetics based on detailed analysis of currents through single channels; the sophistication of this analysis allows very small differences in physiological properties to be detected. However, very few channels have been examined at this level. The remaining part of this essay attempts to describe the development of our current understanding of ion-channel structure, diversity and function. Molecular studies on channel proteins and channel genes have played a vital role in this process.

Ligand-Gated Channels

Channels such as the acetylcholine receptor (AChR) open in response to neurotransmitter binding; i.e., they undergo conformational changes to form an open ion-selective pore across the membrane. Such channels are called ligand-gated channels. They are found at high concentrations in postsynaptic membrane. They open in response to neurotransmitter binding, close in a time-dependent fashion (a process known as "desensitization"), and then take a significant time to "recover" to an activatable state. During the time of one such cycle, neurotransmitter in the synaptic cleft is usually destroyed by a specific hydrolase or removed by a specialized uptake mechanism.

Neurotransmitter receptors are named for the neurotransmitter to which they respond, for example GABA receptors, glycine receptors, acetylcholine receptors etc. The selectivities of ligand-gated channels may differ; nicotinic acetylcholine receptors form channels selective for small cations while GABA receptors are selectively permeable to small anions such as Cl^-. Several receptor subtypes often exist that respond to the same neurotransmitter though they may vary either in ligand-sensitivity or in the exact nature of their response. For example, a family of pharmacologically and biochemically distinguishable GABA receptors, whose different functions are not clearly understood, has been identified in rat (16). In addition to the class of neurotransmitter receptor that forms a ligand-gated channel, there is a large class that binds neurotransmitter molecules but does not itself form a transmembrane pore. Instead, these receptors couple neurotransmitter binding to ion channel molecules in the cell either directly, or via second messenger systems. Examples include adrenergic receptors, muscarinic acetylcholine receptors (AChR), dopamine receptors and serotonin receptors. Different members of this class of receptors may act synergistically or antagonistically upon the same type of ion channel (17). Some of these neurotransmitter receptors have been shown to have roles beyond

membrane excitability; they may control developmental processes such as axonal outgrowth or cell proliferation (18, 19).

The Nicotinic ACh Receptor: A Prototypic Ligand-Gated Channel: The best characterized ligand-gated channel is the nicotinic acetylcholine receptor found in the vertebrate neuromuscular junction. Its physiological properties have been examined in detail at a single channel level from many different biological tissues and these properties have been extensively reviewed (20). Its biochemical characterization has hinged on the availability of a source of tissue with a high concentration of the receptor and on the availability of natural toxins that bind the channel with high affinities. The electric organ of the electric fish *Torpedo californica* is an especially rich source of the receptor; detergent extracts of electroplax plasma membrane contain high concentrations of the protein. Several organisms have evolved toxins that paralyze predators or prey via the acetylcholine receptor. Toxin from the venom of the king cobra *Naja naja* was used for the affinity purification of the AChR. The purified receptor has been biochemically and electrophysiologically characterized by several groups. The purification of the receptor protein, and the generation of antibodies against the polypeptides, has allowed the cloning of the genes encoding the receptor protein (21). Physiological properties of cloned receptor have been studied by the expression of functional receptor proteins in a variety of expression systems including *Xenopus* oocytes and cultured mammalian cells (22, 23). The major molecular properties of the receptor are summarized below.

The endplate acetylcholine receptor is a pentamer composed of four distinct subunits in the stoichometry alpha:beta:gamma:delta, 2:1:1:1 which range in size from 45kD to 60kD (24). In adult skeletal muscle the gamma subunit is replaced by a subunit called epsilon which accounts for physiological differences that have been observed between embryonic and adult AChRs (25). All subunits are about 50% identical to each other in aminoacid sequence and share several structural features. Each polypeptide has 4

hydrophobic domains and one amphipathic domain; these may be membrane spanning regions. It has not been definitively shown whether 3, 4 or 5 of these domains actually cross the membrane; however biochemical evidence suggests that the N-terminus of the alpha subunit is extracellular. The subunits are believed to assemble around a central pore in a pseudosymmetric fashion; this has been confirmed by electron microscopy and by x-ray scattering studies (26, 27).

A variety of experiments, including extensive site-directed mutagenesis, has aimed at identifying specific functional domains in the channel protein. Acetylcholine binds to a site, on the N-terminal extracellular portion of the channel, that has been determined by several independent criteria including site-specific mutagenesis (28). The mechanism by which acetylcholine binding induces an open conformation of the channel is, as yet, unknown. Elegant experiments with channel chimeras, pharmacological agents and site-directed mutagenesis have shown that the second hydrophobic segment (M2) of each subunit most probably forms the lining of the channel pore. These segments contain several serine and threonine residues, that could form a hydrophilic channel lining; mutations in these polar residues affect channel conductance and local anaesthetic binding. It has also been shown that negatively charged aminoacids bordering the M2 segment are at least partially responsible for determining channel conductance (25, 29, 30). There are now several detailed models for the structure of the acetylcholine receptor, but few have been tested to any significant extent. Several groups have been attempting to crystallize the protein; however, none have yet succeeded in making crystals that may be useful for x-ray diffraction studies.

Neuronal ACh receptors are closely related to endplate AChRs. Genes for many of these proteins have been cloned by low stringency hybridizations with endplate AChR cDNA probes. The neuronal AChRs appear to have only two different subunits, alpha and beta, that associate with each other in an unknown stoichometry. At least 3 distinct alpha subunit genes and beta subunit genes have been cloned from rat. These have been shown to

assemble in complex ways with one another, to give many physiologically different AChRs including the bungarotoxin insensitive nicotinic ACh receptor that has been identified in the vetebrate CNS (31, 32). Thus a great deal of heterogeneity may be generated in the nervous system by subunit shuffling. It is possible that more subunits remain to be identified.

Several other ligand-gated channels are similar in structure to the nicotinic AChR. Mammalian genes for GABA receptor subunits have been cloned after affinity purification of the protein with benzodiazepam; the gene for the strychnine binding subunit of the glycine receptor has also been cloned (33, 34). These genes are clearly related in sequence to the AChR. It has been suggested that ligand-gated channels form a related "superfamily;" that all the genes for these proteins have evolved from a single primordial ligand-gated channel. This premise is borne out in part by the sequence of a glutamate receptor gene, cloned by "expression cloning" techniques, that shows little significant sequence similarity to the other members of the ligand-gated channel gene family, but shares the basic structural organization of these genes. There is also some similarity in sequence around the second transmembrane segment, M2, that may be significant (35). The cGMP-gated channel from vertebrate photoreceptors, however, shows no similarity in sequence or in structural organization to the ligand-gated channel superfamily. Instead it shows some limited similarities to cGMP binding proteins and might have an independent evolution (36). The recently cloned IP_3-gated calcium channel in endoplasmic reticulum and the calcium release channel in muscle sarcolemma show significant homology to each other in some regions; these two genes also appear to define a separate family (37, 38). Most current efforts in the molecular analyses of ligand-gated channels attempt to a) correlate specific aminoacid sequences with particular channel functions; b) correlate electrophysiologically identified receptors in the nervous system with specific molecularly identified receptors; or c) clone new genes for neurotransmitter receptors.

Voltage-Gated Ion Channels

Ion channels that open in response to changes in membrane potential are called voltage-gated channels. They are highly selective for specific ions and are named for the ions that they conduct. Most voltage-gated channels open in response to membrane depolarization although a few, such as the anomalous rectifier, open on membrane hyperpolarization. After opening from the resting state, some channels close in a time-dependent fashion into an inactive state and then take a significant time to recover to the activatable (resting) state. The best example of such channels is the sodium channel fron squid giant axon. Non-inactivating channels (for example delayed-rectifier type potassium channels in squid axon) remain open until membrane repolarization makes them adopt the closed conformation.

Voltage-gated sodium channels, potassium channels, calcium channels and chloride channels have been identified in most multicellular organisms. Each of these categories includes several diverse channel types that vary in a variety of parameters, including voltage-sensitivities, rates of opening, open times, conductances, rates of inactivation, rates of recovery from inactivation, sensitivities to Ca^{++} and other divalent cations, sensitivities to second messengers and to various pharmacological agents. Many of these specific channel-types are found in a wide range of phyla. The next two sections discuss the structure and diversity of different types of sodium- and potassium-selective channels.

Sodium Channels:

Biochemical Purification and Functional Reconstitution:

Physiologically characterized sodium channels have many features in common. They have high selectivity for sodium ions over all other physiological cations ($Li^{+}> Na^{+}> NH_4^{+}> K^{+}>> Ca^{++}$) and rates of conductance higher than 10^7 ions per molecule per second (39). Sodium channels activate rapidly in response to membrane depolarization (t1/2 for sodium conductance rise in the squid axon is approximately 0.5ms at 10°C) and

then inactivate in a time-dependent manner (t1/2 for inactivation is approximately 2ms). The channels are then insensitive to voltage during a quiescient (inactive) period. The membrane must then be hyperpolarized for many milliseconds before the channels recover their earlier properties. This cycle of activation, inactivation, and recovery from inactivation is observed in most sodium channels. Molecular models of sodium channels strive to explain these basic processes.

The logical first step in the study of the molecular structure of sodium channels was the biochemical purification of the protein. In 1973, the tetotrotoxin-binding component of the garfish olfactory nerve was successfully solubilized using nonionic detergents. However the toxin-binding activity of the solubilized preparation was too unstable to be used as an assay for more extensive purification. The addition of phospholipid and calcium to the detergent-solubilized sodium channel was later found to stabilize dramatically the toxin-binding activity of the channel (14). The saxitoxin receptor has now been purified to a high degree of homogeniety from several sources including electric eel electroplax, several mammalian skeletal muscle and mammalian brain. Table 2 shows the molecular weights, subunit compositions and carbohydrate contents of sodium channels purified from each of these sources (14). It is apparent that all sodium channels include a large heavily glycosylated subunit (alpha) containing the TTX binding site, and in some tissues include one or two smaller subunits. In rat brain the smaller subunits are called ß1 and ß2 respectively; ß2 is attached to the alpha subunit by disulfide bonds while ß1 is noncovalently attached.

Purified ^{3}H TTX (or ^{3}H STX) receptors from rat brain, eel electroplax and rat skeletal muscle have been reconstituted into lipid bilayers. Single-channel currents from these reconstituted channels show relatively normal physiological characteristics. The channels are sensitive not only to TTX, but also to unrelated toxins such as veratridine and batrachotoxin that affect native sodium channel in characteristic ways. Thus, the TTX receptor appears to include most of the entire functional sodium channel. An observable

difference is that most purified sodium channel preparations show no activity in lipid bilayers in the absence of BTX, but then behave like BTX-modified channels with little inactivation and a low threshold of activation. The reason for this is phenomenon is unknown. However similarly purified channels reconsituted into multilamellar lipid vesicles show channel activity in excised patches even in the absence of BTX (40). The most significant knowledge gained from these studies is a) that the purified proteins are indeed identical to the sodium channel; and b) that the large subunit of the sodium channel is capable of independently forming a voltage-gated, sodium-selective pore with much of the pharmacological sensitivities displayed by sodium channels in excitable membrane. The successful purification of sodium channels paved the way for the molecular cloning of the sodium-channel gene, that is described later in this chapter.

Diversity of Sodium Channels:

Despite popular belief to the contrary, several independent experiments began to suggest that sodium channels were a diverse population of channel molecules. The major lines of evidence were from three sources: a) pharmacological sensitivities of sodium channels; b) immunological reactivities of sodium channels; and c) electrophysiological properties of sodium currents in varied organisms and excitable tissue.

Pharmacological and immunological evidence: TTX was the first means of demarcating two classes of sodium channels. TTX-resistant sodium channels were described in cardiac muscle in 1976 (41). Similar channels were found in several other biological tissues including denervated vertebrate skeletal muscle and in immature nerve and muscle membrane (42, 43). Other toxins, such as μ-conotoxin from *Conus geographus,* were used to distinguish TTX-sensitive channels in brain from those in skeletal muscle (44). Yet another toxin from scorpion venom preferentially bound sodium channels on muscle membrane while showing little affinity for those in the T-tubular system (45). Monoclonal antibodies raised against purified sodium channels from skeletal

muscle were used to identify three subpopulations of sodium channels in adult rat skeletal muscle (46). These antibodies distinguished channels in the sarcolemma from channels in the T-tubular membrane. Sodium channels in the T-tubule system of slow-twitch muscle fibers could also be distinguished from those in fast-twitch fibers. This analysis also showed that channel subtypes that could be distinguished by one antibody, were not distinguished by others. Thus, the subtypes shared some common epitopes, and probably represented distinct, but related, proteins.

Electrophysiological evidence: Differences in electrophysiological properties of sodium channels have been detected both by voltage-clamp and by patch-clamp analysis. Unusual sodium channels that differed significantly from the classical Hodgkin and Huxley sodium channel were discovered in many tissues. An aesthetically pleasing example, that also serves to demostrate a distinct biological function for such sodium channels, is in the giant axon of the worm, *Myxicola* (47, 48). In response to touch, the escape response of *Myxicola* is mediated by an action potential through its giant axon. However, upon repeated stimulation at frequencies greater than 5Hz, no escape response is generated by later stimuli. This behaviour, called "adaptation," is mimicked when a *Myxicola* is held in the hand: "when first picked up (it) will twitch violently, but after several twitches the worm will lie motionless in the hand." Voltage-clamp analysis of currents carried by the giant axon showed that sodium channels recover very slowly from inactivation (greater than one second for 90% recovery). At high frequencies of stimulation fewer sodium channels are available to respond to succeeding stimuli and hence the axon is incapable of sustained firing. Thus, this unusual sodium channel forms the basis for frequency-dependent adaptation of the giant axon. It has not been shown, however, due to a regrettable lack of interest in *Myxicola* physiology, that *Myxicola* has other classes of sodium channels.

Electrophysiogical evidence exists for diverse populations of sodium channels in the same organism. In squid giant axon it has been demonstrated that there are two

populations of sodium channels. The major class comprises more than 95% of the sodium channels in the axon and has been described by Hodgkin and Huxley; the minor class called "threshold channels" activates at potentials close to the resting potential and has a very slow rate of inactivation. Thus these channels dominate behaviour of the axon membrane in the threshold region for action potential initiation (49). In rat, electrical conductances in several different tissue types have been examined. A noninactivating ($t_{1/2}$ > 100ms) sodium channel has been identified in the soma of cerebellar Purkinje cells, cells with very complex firing patterns. When calcium conductances are blocked, the action-potential waveform in these cells includes a transient sodium dependent spike probably mediated by Hodgkin and Huxley type sodium channels; this is followed by a smaller, prolonged sodium-dependent plateau, during which a noninactivating sodium channel is active along with various potassium conductances. Both inactivating and noninactivating sodium conductances are TTX sensitive. The precise contribution of the noninactivating sodium channel to the normal physiology of the cell is not clear; the current is clearly seen only when calcium conductances are blocked (50).

Single channel records from rat myotubes and myoblasts shows the coexistence of two classes of sodium channel; a TTX-sensitive channel with a 12pS conductance that activates at more depolarized potentials than a TTX-resistant channel with a smaller conductance (10pS). The relative proportions of the two channel types changes during muscle development. The TTX-resistant channels may serve a similar function in muscle membrane as the threshold channels in squid axon (51). Three different types of TTX-sensitive sodium currents have been described in membrane patches from rat heart ventricular cells. These appear to have similar conductances but vary most obviously in their rates of inactivation. Rates of inactivation have been used to classify them as "fast," "slow" and "ultraslow" (τ = 0.6ms, 13.9ms and 130ms respectively, at -30mV). It was suggested by the authors that these different currents reflected different "modes" of a given sodium channel molecule rather than different sodium channel subtypes. This issue was not

resolved, however, as all the patches used in the studies contained multiple (4-50) channels (52).

A different type of sodium channel has also been found in glia. Type 1 atrocytes express a TTX-sensitive sodium channel with slower activation (long latencies to first opening in single channel records) and slower inactivation (longer mean open times) than neuronal TTX-sensitive channels. In addition their voltage dependences of activation and inactivation are shifted in a hyperpolarizing direction; this may be an adaptation for glial function as glia have resting potentials more negative than neurons (53).

Multiple sodium channels types have been identified in a variety of other preparations. The characterization of these channel types and their physiological functions remains a subject of intense research. A more fundamental problem in the mid 1980s was the mechanism by which these different channel activities were generated. Were they alternate modes of the same protein; if so how were these modes generated in the cell? Or were they independent proteins whose synthesis and assembly were under complex spatial and temporal regulation? The other question was one pondered for many years: what is the molecular architecture of the sodium channel?

Molecular Genetics of Vertebrate Sodium Channels

Sequence of the Sodium Channel from *Electrophorus electricus*:

The first sodium channel gene to be cloned was from electric eel electroplax (54). Protein purified from this tissue was digested with trypsin and the N-terminal sequence of purified tryptic fragments was determined by protein microsequencing. Complementary DNAs from the sodium channel gene were isolated by a combination of immunological screening of an expression library with hybridizations to radiolabelled oligonucleotides corresponding to known peptides. The deduced aminoacid sequence of the channel is briefly described below.

The eel sodium channel is composed of a single large polypeptide 1820 aminoacids in length. This corresponds roughly with the molecular weight of the deglycosylated, purified channel. The sequence includes 10 potential sites for N-glycosylation, sufficient for the addition of sugar groups that comprise 29% of the mature protein. The absence of an N-terminal signal sequence suggests that the N-terminus of the polypeptide is intracellular.

The most striking feature of the protein is the presence of four internal repeats that are roughly 50% identical to one another. The repeats are about 250 aminoacids in size; they are referred to, in order from the N- terminus, as "homology unit" or "homology domain" I, II, III and IV. Each homology domain contains six characteristic hydrophobic segments about 24-38 aminoacids in length. The segments (S1-S6) are capable of spanning the plasma membrane. S5 and S6 are very hydrophobic segments flanked by charged residues; these characteristics are typical of membrane spanning segments. S1, S2 and S3 generally contain a few charged residues. Negatively charged groups predominate in S1 and S3 while both positive and negative charges are found in S1. If an α helical structure is assumed for these segments, the charged side chains cluster on one phase of the helix. Thus each of these segments is capable of forming an amphipathic helix in which the polar residues are shielded from lipid membrane. The S4 segment in each repeat is positively charged with 4-8 arginine or lysine residues; while these basic aminoacids are located at every third position, the other residues in S4 are hydrophobic. Thus, if one assumes a 3_{10} helical conformation all the positive charges line up on one phase of the helix. In an α helical conformation the charged groups are arranged in a spiral; in a ß pleated sheet they extend alternately to opposite sides of the peptide backbone. Negatively charged residues are found between S5 and S6 of all repeats, as also in the region between homology domains II and III.

These features of the eel sodium channel sequence clearly suggested a rough structural organization for the channel. The individual homology domains are arranged

pseudosymmetrically in the channel; thus there are an even number of transmembrane segments (4 or 6) per homology domain. The unusual positively charged S4 segments may sense and respond to changes in membrane potential (54). Several detailed models for sodium channel structure were spawned from the sequence of the eel sodium channel (55, 56, 57, 58). There is however a paucity of data to confirm or refute details of the models; for this reason, this essay will only discuss features of the models that are supported by experimental data.

Structural models for sodium channels were refined by the isolation and characterization of a family of closely related rodent sodium-channel genes. Workers in Kyoto used eel sodium-channel cDNA probes to isolate and characterize three distinct, sodium-channel genes expressed in rat brain (RSC1, RSC2 and RSC3) (59). The sequence of a fourth sodium channel gene specifically expressed in skeletal muscle, SKM1, has recently been reported (60). An independently isolated rat brain sodium channel gene, RatIIA shows greater than 99% DNA sequence identity to RSC2; it appears likely that it is the same gene as RSC2 isolated from a different strain of rats (61).

The basic structural organization of the rat sodium channels is identical to the eel channel; each has four homology domains with similar characteristic features. The aminoacid sequences of the channels are extremely similar to one another. The overall homology (percentage sequence identity) among the rat channels is about 75%; this figure exceeds 90% within the four homology domains. Significant differences are present in regions linking the internal repeats to each other. Some features, such as the clustered negative charges between homology domains II and III, present in the eel sequence, are absent in the rat sodium channels; they probably do not serve key functions assigned to them in some models (54, 55). Additionally, the rat brain channels have a large insertion of about 170 residues with multiple consensus sites for phosphorylation by A-kinases between homology domains I and II; this insertion is absent in the eel channel and also in

SKM1. Significantly, a short, lysine-rich linker region between domains III and IV is remarkably well conserved among all known sodium-channel sequences.

Physiological Properties of Cloned Sodium Channels:

One of the most important requirements for structure-function correlations to be made in a protein, is the ability to study the activity of the protein, once its sequence has been estabilished. As the eel sodium channel gene could not easily be engineered to express functional protein, RSC2 was the first sodium channel gene to be successfully expressed in a heterologous expression system (62). In this commonly used method, *in vitro* transcribed mRNA was microinjected into stageV-stageVI *Xenopus* oocytes. Two-microelectrode voltage clamp was used to monitor channel activity in the oocyte membrane, two days after the injection. In these assays, RSC2 mRNA induced voltage-dependent sodium currents that activated and inactivated rapidly; the currents were abolished by 1μM TTX but were insensitive to μ-conotoxin. This experiment served several purposes: a) it showed that the large subunit of the channel could independently form sodium channels in *Xenopus* oocytes; b) it showed a method for the detailed characterization of an isolated channel type; c) it also provided a method for the study of mutant channels modified *in vitro*, a procedure critical for correlating channel structure with its function. Although it is not ideal to study a channel in a heterologous environment, the oocyte expression system has been extremely useful not only for the characterization of cloned sodium channels (60, 62, 63), but also for the study of channels in RNA from specific tissues, and for the cloning of new channel genes (64, 65, 66).

RSC3, RatIIA and SKM1 channels have also been successfully expressed in *Xenopus* oocytes. No significant differences have been reported between RSC2 and RSC3 currents (67). Though the first report of the physiological properties of RatIIA indicated a significant difference from RSC2 in the voltage dependence of activation, this difference was later attributed to an inadvertant cloning artifact that altered a critical leucine residue in

the channel (61, 68). Thus no real physiological differences have yet been detected between functionally-expressed brain sodium channels. The SKM1 current shows a dramatic difference in pharmacology; it is insensitive to 100nM TTX and is completely blocked by 5nM μ-conotoxin.

Voltage-sensitive sodium currents may be induced in *Xenopus* oocytes by the injection of total mRNA prepared from rat brain. These currents inactivate more rapidly that currents induced by the injection of *in vitro* transcribed mRNA (cRNA) from cloned channels. Both currents may be made indistinguishable if small molecular weight RNA (2-4kb), fractionated from total brain, is coinjected into the oocytes with cRNA. This observation suggests a role in inactivation for small proteins, presumably the small subunits of rat brain sodium channels (63).

Structure-activity correlations in sodium channels:

There is a surfeit of models of hypothetical sodium channel structures and of correlations of modelled structures with specific channel functions (58, 69, 70). These models will not discussed in this essay. Instead, the essay will briefly review the sparse experimental data that exists in this area of research. Most of these data associate specific aminoacid residues or specific regions in the protein with particular channel functions such as activation or inactivation.

Activation and Inactivation: It has been apparent for several years that voltage-gated sodium channels contain charged residues located close to or within the membrane. Depolarization of membrane could cause this "activation gate" to move, triggering conformational changes in the protein (69, 71). The positively-charged S4 segment of the sodium channel has all the features expected of a voltage sensor; all popular models suggest that S4 resides in the membrane and associates with negatively charged groups in other transmembrane segments. To test the role of S4 as a voltage sensor, a series of mutant sodium channel cDNAs were constructed *in vitro*; they carried one or more sequence

alterations in the S4 segment of the first homology domain. Channels expressed from these mutant cDNAs were assayed in *Xenopus* oocytes for altered physiological function. A decrease in the steepness of the potential dependence of activation was observed with decrease in positive charge on the S4 segment. Such a result is predicted by mathematical models of voltage-dependent activation gates. The experiment has been interpreted as a confirmation of the hypothesis that S4 is the voltage sensor in voltage-gated channels and that positive charges in S4 constitute (at least part of) the gating charge in sodium channels (72).

The intracellular perfusion of squid giant axon with various proteases effectively removes the inactivation process of the sodium channel. This was first reported with pronase, a mixture of several distinct proteinases with broad specificity (15, 73). The experiment was repeated with proteases purified from crude pronase, and also with other pure preparations of proteases such as trypsin, papain, ficin and α-chymotrypsin.Their effectiveness of the different enzymes in blocking inactivation was correlated with the enzyme specifities. Based on this analysis, it was proposed that an intracellular peptide including lysine or arginine residues was likely to be involved in inactivation (74).

A lysine rich region between homology domains III and IV is very highly conserved in different sodium channels. Antibodies to this peptide were generated and were perfused into myoballs (colchicine treated skeletal muscle cells) that were analyzed under voltage clamp. The antibody was found to reduce specifically the rate and extent of channel inactivation (75). In parallel experiments, sodium channel cDNA was cleaved between homology domains III and IV. RNA transcribed in vitro from the two cDNA fragments were coinjected into *Xenopus* oocytes. Functional but non-inactivating sodium channels were expressed on oocyte membrane. These experiments to identify sequences involved in inactivation, have all pointed toward the same conserved intracellular segment. However, other sequences are also likely to be involved, as judged by the effect of some extracellularly applied ligands including antipeptide antibodies and peptide neurotoxins

from scorpion, sea anemone, coral and snail. These ligands affect inactivation in different ways (76). There is little known about the molecular mechanism of channel inactivation. Indeed, I do not know of an enzyme with a function truely analogous to inactivation. Autoregulation of enzymes such as many protein kinases, or subunit dissociation of G-proteins on activation, are the closest analogies to this process.

The Channel Pore: Sequences close to the sodium-channel pore have been identified in an aesthetically pleasing fashion. It has been known for a long time, that TTX-sensitive channels may be made insensitive by treatment with carbodiimides or trimethyloxonium, reagents that modify carboxylic acid groups. This process concurrently reduces single channel conductance (39). Thus the TTX binding site is likely to be intimately associated with the conducting pore. Point mutants have been constructed in the rat sodium channel RSC2, that alter specific acidic residues on presumed extracellular regions of the sodium channel. One of these changes, glutamic acid residue 387 to glutamine, causes a greater-than-1000-fold reduction in binding affinity for TTX. The same change also reduces the unitary conductance of the sodium channel. This acidic residue lies between S5 and S6 of homology domain II (77). This region has been proposed to form the channel pore in at least one structural model (57).

The most impressive feature in sodium-channel function is its high selectivity for sodium combined with a conduction rate close to that expected for free diffusion of sodium ions. Mutant channels that affect ion-selectivity have not yet been constructed; no convincing structural model for this function exists to direct the mutagenesis experiments. In some experiments specific, amphipathic peptide fragments from the sodium channel have been reconstituted into lipid bilayers, and their ability to assemble into channels has been assayed. Ion channels with several different conducting states have been detected in these experiments, presumably due to different sized aggregates of the amphipathic peptides. It appears clear from these studies, that any sequence capable of forming an amphipathic helix can also form very weakly ion-selective channels. Twenty-two-

aminoacid peptides from either S3 or S4 can independently form ion channels in lipid bilayers (78, 79). The former form voltage-insensitive, cation-selective channels with no selectivity for sodium over potassium; the latter form voltage-gated and, surprisingly, cation-selective channels with no preference for sodium over potassium. These experiments, while interesting in their own right, do not determine the real sodium channel pore lining. There is no reason to assume that the peptide sequences that line the pore interact directly with lipid membrane.

Current research in vertebrate sodium channels:

Several groups have been involved in identifying new sodium channel genes using low stringency hybridization and PCR techniques on cDNA from different tissues. At least 8 different sodium channel α-subunit genes from rat have been partially characterized in several laboratories. One of these is reportedly specific to cardiac muscle.The cellular or subcellular distributions, physiological properties and genetic map positions of these genes are subjects of active research. A large collaborative effort to identify other sodium channel subunits is in progress; this has not yet been successful. A few groups have been involved in studying transcriptional regulation and post-translational modification of sodium channels; processes that could conceivably serve to modulate, *in vivo*, cellular responses to stimulation (discussed in greater detail in the next section on potassium channels). Many groups have been studying the structure of sodium channels with several different methods and objectives. Detailed study of sodium channel structure is completely justified by the general paucity of structural information on membrane proteins. The more commercial purpose of this study is the development of sodium-channel subtype-specific drugs and the prospect of "channel engineering" (80).

Genetic methods have been used to identify and study genes involved in invertebrate sodium-channel function. These experiments are confined to *Drosphila* and are

briefly discussed at the end of this essay, and in Chapter 2. The next section will deal with potassium channels, their physiological analysis and molecular characterization.

Potassium Channels

Potassium-selective channels are probably the first ion-selective channels to have evolved and are ubiquitously found in all phyla that have been examined. They have had more time to diversify than other channel types and, consequently, have been recruited to serve several diverse physiological functions in different cell types. Specific blends of potassium channel types in cell membrane largely determine the various action potential waveforms and neuron-specific firing patterns that have been observed in complex nervous systems (69, 81, 82). Potassium channels may be categorized according to their physiological properties into four groups that may partially overlap: a) voltage-gated potassium channels; b) inward rectifiers; c) Ca^{++}- activated potassium channels; and d) neurotransmitter- and second messenger-regulated potassium channels. Only voltage-gated potassium channels are discussed in this essay; the other potassium-channel groups are mentioned briefly (81, 83).

Voltage-gated potassium channels:

Nomenclature: Voltage-gated potassium channels have been roughly grouped into two categories: the "delayed rectifier" (K) channels and the "A" channels. The prototypic delayed rectifier is that described by Hodgkin and Huxley in the squid giant axon. The potassium current in squid axon activates with a delay on membrane depolarization, and reaches its peak more slowly than the voltage-dependent sodium current. The current inactivates relatively slowly (τ >500 ms) at potentials greater than the threshold for action-potential generation; it is also relatively insensitive to intracellular calcium concentration. All similar potassium currents responsible for action potential repolarization are called delayed rectifiers or K type currents.

The prototypic A current was first described in the soma of a molluscan bursting neuron. The outward potassium current in this cell can be separated under voltage-clamp into two components with clearly distinguishable properties. One component is similar to the classical delayed rectifier; the other activates rapidly at subthreshold potentials.The subthreshold current inactivates rapidly with a steep dependence on membrane potential; the current is almost completely inactivated by voltage prepulses near the action-potential threshold. In addition, the inactivating current is more sensitive to 4AP and less sensitive to TEA than the K current. These physiological properties, coupled with a lack of dependence on intracellular calcium, are characteristics that define A channels. A-type channels dominate the behaviour of the neuron at subthreshold potentials; in bursting neurons, they control the interspike interval (84). The cellular functions assigned here to A type and delayed rectifier channels are often shared by other channel types, most significantly by calcium-activated potassium channels.

Physiological diversity: Since Connor and Stevens' analysis in 1971, several voltage-gated potassium channels have been physiologically characterized from a variety of excitable tissue; many of these have properties intermediate between the classical delayed rectifier and A-type channels. As there is often no clear distinction between these two groups of ion channel, very similar channels in different preparations have been labelled as either A type or K type, by different investigators (81). Thus voltage-gated potassium channels may traverse the entire spectrum from classical A type to classical delayed-rectifier type channels. It is possible that the present classification into A type and K type channels is a historical legacy, that currently gives us little insight into the properties and functions of potassium channels.

Several subtypes of voltage-gated potassium channels have been identified in the same organism, using voltage-clamp and patch-clamp techniques in the manner described for sodium channels. Voltage clamp analysis of the frog node of Ranvier has revealed three

distinct voltage-gated potassium currents: a low threshold, fast transient current (f1), a higher threshold fast transient (f2) and a slowly activating non inactivating component (s) (85). These observations have been partially supported by fluctuation analysis of potassium conductances in large patches from the node of Ranvier. However the latter study has been interpreted as evidence for multiple conducting states of the same channel (86); this interpretation is hard to reconcile with the clear differences in sensitivities to peptide toxins between f1, f2 and s channels reported by Dubois (85). The macroscopic delayed-rectifier current changes during maturation of *Xenopus* spinal neurons. The developmental regulation of two populations of voltage-gated K^+ channels with different unitary conductances underlies this phenomenon. It has been reported that in addition to the densities of both channel types increasing during maturation, the kinetics of the larger conductance type also changes; the molecular basis for this phenomenon is not known (87). Instead of an overwhelming, comprehensive discussion of different voltage-gated potassium channels, it probably suffices to hint at the diversity of these channels by listing the characteristics of voltage-gated potassium channels in rat, the organism best studied by cellular physiologists. Table 3 (that draws extensively from Rudy, 1988) shows the various channel types and their characteristics (81).

Several problems are apparent in trying to gauge the full extent of potassium channel diversity from independent characterizations of potassium currents in various cell types. 1) The membranes of different cells have different compositions. It has been shown that lipid composition can affect both the unitary conductance of channels, and their activation and inactivation properties (81, 88). It has also been shown that channels may associate with cytoskeletal elements in some tissues and such association could have effects on physiological properties (89). 2) Different procedures and protocols have been used by different groups. For example: pharmacological experiments performed by Dubois on the node of Ranvier potassium channels were not repeated by Conti et al. in their later study of the same system, or by Standen et al. in their study on frog skeletal muscle channels. 3) It

is possible that the same channel type has multiple functional states that may vary widely depending on factors such as temperature or surrounding medium (86). In this scenario, diversity of functional channels does not really represent molecular diversity of channel proteins. 4) Macroscopic potassium currents are not easily resolved into separate components. Thus, potassium channel components analyzed by voltage clamp may easily be mixtures of similar channels that are later resolved by more sophisticated methods. However, even in the face of these technical problems, it is clear that voltage-gated potassium channels are a diverse family of proteins that vary widely in their voltage dependencies, conductances and pharmacological sensitivities. Potassium channels may also be modulated differently by second messenger systems and this modulation might add to the observed diversity of voltage-gated channels.

Pharmacology and biochemistry of voltage-gated K+ channels:

The biochemistry of potassium channels is yet in its infancy. Until very recently, there were no specific high-affinity ligands (with K_d values in the nanomolar range) that could be used for the purification of voltage-gated potassium channels. In the 4 or 5 years past, several naturally ocurring peptide toxins that bind voltage-gated potassium channels have been characterized. These include dendrotoxin (DTX) and ß bungarotoxin (ß-BTX) from snake venom; mast cell degranulating peptide (MCDP) from bee venom; and noxius toxin (NTX) {and possibly charybdotoxin (CTX)} from scorpion venom. Several other crude preparations of venom from a variety of sources have also shown effective blockage of voltage-gated potassium currents. These toxins have been used more for the characterization of physiologically identified potassium currents than for the biochemical purification of potassium channels. In general, each of these toxins blocks several different voltage-gated channels that have a wide range of physiological properties: for example, DTX blocks a low threshold, fast transient, potassium current in rat hippocampus, in addition to a slowly activating, non-inactivating current in guinea pig dorsal root ganglion

and an unusual, fast-activating, non-inactivating current in rat visceral afferent neurons (90). This pharmacological evidence suggests that voltage-gated potassium channels may be a family of structurally-related proteins. Toxins that bind non-voltage-gated potassium channels have been much better characterized. Several drugs used in the treatment of cardiovascular disorders, and also in the treatment of a form of diabetes act upon potassium channels; biochemical studies of these channels is a very active area of research that is out of the scope of this essay (90, 91, 92).

Dendrotoxin receptors from brain have been biochemically purified. While it remains possible that this is a heterogenous mixture of polypeptides, a single band 76000-80000 daltons in size has been shown to contain DTX, MCDP and ß-BTX binding sites; specific binding of ^{125}I-labelled MCDP and ^{125}I-DTX is inhibited by ß-bungarotoxin. A polypeptide 38000 daltons is copurified in this procedure. It is believed that the K^+ channel is a multimer of these subunits with yet unspecified stoichometry (93).

Molecular genetics of voltage-gated potassium channels

Primary structure of voltage-gated potassium channels:

An elegant combination of genetic, electrophysiological and molecular studies on the *Shaker* locus of *Drosophila melanogaster* led to the first cloning of a voltage-gated potassium channel gene. Several distinct mRNAs are generated by alternative splicing at the *Shaker* (*Sh*) locus. At least six of them are capable of directing the synthesis of potassium channels when independently microinjected into *Xenopus* oocytes; the nature of these different currents are discussed later. The mRNAs follow a simple pattern in which variable 5' and 3' ends are spliced onto a central "constant" region. The constant region of functional *Shaker* polypeptides is about 400 aminoacid residues in size; the variable amino domains range from 31 to 61 residues; the variable carboxyl domains are between 170 and 240 residues. Several features of the sequence of *Sh* channels are discussed below (94, 95, 96).

The four functional amino domains of the different *Sh* channels show no similarities to one another or to other known sequences; there are no obvious distinctive features in these sequences. The two carboxyl regions (that have been shown associated with functional channels) begin at a putative transmembrane segment (H6/S6). There is considerable homology between the two regions until a little after this last putative transmembrane segment; after a conserved glutamine rich region, the sequence diverges completely until the last three aminoacid residues (Thr-Asp-Val). The molecular significance of these sequences is yet unclear.

The constant region shows many more distinctive features. It has five (or, in some models, six) putative transmembrane segments that are clustered within about 250 aminoacids. The fourth segment (S4) resembles the putative voltage sensor of the sodium channel, in containing several (7) iterations of the motif Arg/Lys-X-Y (where X and Y are hydrophobic aminoacids). Thus, there are compelling similarities between the *Shaker* constant region and one homology domain of a voltage-gated sodium channel. Five iterations of a leucine heptad (a leucine residue at every seventh position) occur adjacent to and downstream of the S4 segment (97). Such leucine heptad repeats have been shown to be involved in subunit association and protein-protein interactions in a family of DNA binding proteins (98). Less striking, but distinct, leucine heptad repeats are also found at a similar location in voltage-gated sodium- and calcium-channel sequences.

Due to the several observed sequence similarities between voltage-gated potassium and sodium channels, topological and structural models for potassium channels have drawn heavily from models for sodium channels. It is believed that voltage-gated potassium channels are a multimeric (probably tetrameric) assembly of smaller polypeptides analogous to single homology domains of the sodium channel. The idea that potassium channels are a multimeric assembly of subunits is supported by genetic data from *Drosophila* (99, 100, 101).

Functional properties of *Shaker* channels in oocytes:

Eight *Shaker* cDNAs have been individually expressed in *Xenopus* oocytes. These experiments have served several functions. Most importantly, they establish that a single *Shaker* polypeptide may assemble into a functional potassium channel, at least in *Xenopus* oocytes; thus the *Shaker* locus may encode at least eight homomultimeric channels. The properties of the different currents indicate specific functions that reside both in the constant domain and in the different amino and carboxyl domains (102, 103, 104, 105). All *Shaker* channels are identical in potassium conductance, selectivity, voltage dependence of activation and inactivation, and in sensitivity to 4AP and CTX. These functions presumably reside in the constant region - in sequences common to all *Sh* channels. However, cDNAs derived from the *Shaker* locus encode potassium channels that vary widely in their inactivation properties. When all combinations of amino and carboxyl domains are analyzed, it appears that the amino domains determine the rate of inactivation of the channels, the carboxyl domains appear to be primarily responsible for the rate of recovery from inactivation. Table 4, taken from Iverson and Rudy (1990), summarizes the properties of six different *Sh* cDNAs. The molecular basis for these functions is yet unknown. Significantly, both fast inactivating, transient potassium channels and very slowly inactivating, delayed-rectifier type potassium channels arise from the *Shaker* locus. This suggests that, in general, the two types of channel may be evolutionarily and structurally related. This is in accord with the pharmacological evidence, and the physiological descriptions of different potassium channels that have been discussed in the earlier sections.

Eight distinct, homomultimeric channels are generated by alternative splicing at the *Shaker* locus. An exciting possibility is the formation of heteromultimeric channels, each with different properties. This would result in an almost absurdly large number of potassium channels generated by subunit shuffling. Unpublished results from our laboratory and others have shown conclusively that heteromultimeric channels, with

distinctive properties, are formed in *Xenopus* oocytes coinjected with mRNAs from two independent cDNAs; the *in vivo* significance of this finding is intriguing, but unclear.

The *in vivo* significance of channel properties studied in any heterologous system may, of course, be debated. The reservations about these data are especially justified because differences have been observed between cloned channels expressed in different cell types. *Drosophila Shaker* channels in *Xenopus* oocytes are CTX sensitive, while in transgenic *Drosophila* muscle (in which the wild-type *Shaker* gene has been deleted), the channels are CTX insensitive; the reason for this dramatic difference is not known but it may result from different post translational modifications, or from other, as yet unidentified, potassium channel subunits present in one of the cell types (106, 107). Rat sodium channels (RatIIA) expressed in frog oocytes differ from the channels expressed in CHO (Chinese Hamster Ovary) cells - this difference has been ascribed to the availability, in the hamster cells, of small sodium channel subunits (108).

Structural models for *Shaker* channels:

Voltage-gated potassium channels were cloned and sequenced in 1987; their similarities to voltage-gated sodium channels were immediately obvious. At about the same time, the sequence of a voltage-gated calcium channel was reported (109). The calcium-channel sequence was very similar to the sodium channel, both in the overall organization into homology domains, and in the organization within the homology domains. Thus, the molecular evidence suggests that these voltage-gated channels belong to a evolutionarily related family; this was predicted by Hille on the basis of a systematic analysis of channel properties and their distributions through different phyla (69). Although there are no published structural models for the potassium and calcium-channel proteins, most current thinking about their structures derive from models for the sodium channel. Thus, voltage-gated potassium channels are thought to be tetramers of small subunits, each subunit analogous to a sodium-channel homology domain. The activation gate is presumed to be

S4, and preliminary mutagenesis experiments of positive charges in S4 confirm that voltage-dependent activation is altered by mutations in this region (110). The amino domains have been associated with channel inactivation; preliminary studies have shown that a 20 aminoacid peptide in this region, that contains some essential positively-charged residues, is necessary for channel inactivation. Deleting these residues from a rapidly inactivating *Shaker* channel converts the channel to an essentially non-inactivating delayed rectifier. Here again, there is some analogy between sodium and potassium channels. Interestingly, the free peptide, applied to the intracellular surface of the mutant, non-inactivating *Shaker* channel, blocks the open channel in a manner similar to channel inactivation.

Charybdotoxin has been shown to block calcium-activated potassium channels by binding at a site close to the extracellular mouth of the channel pore. CTX has also been reported to block *Sh* channels, and a glutamate residue between S5 and S6 has been implicated in the channel association with the toxin (111, 112). This residue, suggested to be at the mouth of the channel pore, is analogous to the glutamate residue in sodium channels implicated in TTX binding (77). One structural issue, that is currently better studied in potassium channels than in sodium channels, is the role of the "leucine zipper," the leucine heptad repeat that occurs shortly after S4 in the channel sequence. Alterations of single leucine residues, to other hydrophobic residues such as valine, cause profound (up to 100mV) shifts in the voltage dependence of activation (113). Similar results have been seen in the sodium channel where one leucine residue in zipper motif was inadvertantly altered (68). While this indicates a role for the leucine residues in voltage-dependent behaviour, the exact role of the leucine repeats is far from established.

Current research in voltage-gated potassium channels:

Several groups have been using a combination of molecular and electrophysiological techniques to study potassium channel structure. Voltage-gated

potassium channels, because of their smaller size, offer some technical advantages over sodium channels. The eventual goals of these efforts are a basic understanding of channel structure, and the more commercial possibilities of channel engineering and drug-design. Several laboratories, including our own, have attempted to isolate and characterize other voltage-gated potassium channel genes from both vertebrate and invertebrate systems. The main goals of these studies are a comprehensive description of voltage-gated potassium channel diversity, and an effective correlation of cloned channels with those implicated in specific physiological functions. While there has been considerable progress on this front over the last two years (this is reviewed briefly in Chapter 3), it remains a very active area of research.

The molecular biology of potassium channels has hitherto lagged behind studies on sodium channels and acetlycholine receptors. The main reason for this lag has been the absence of high-affinity ligands to purify and characterize the channel proteins. These ligands are currently being developed at a fast pace; at least three of these DTX (that binds a class of voltage-gated potassium channels), and CTX and apamin (that bind non-overalpping classes of calcium activated potassium channels with high affinity) are now being used for the purification of potassium channels. Our current molecular knowledge of potassium channels derives exclusively from elegant genetic studies that are feasible only in *Drosophila*, among higher eukaryotes. These genetic methods are also currently being used to characterize and isolate new genes involved in potassium channel function.

Drosophila: genetics of neural excitability:

In general, *Drosophila* offers several advantages for the study of genes involved in nervous system function. It has a complex nervous system with diverse ion channel and neurotransmitter populations; it is, therefore, a suitable organism in which to study channels and neurotransmitter receptors. Its short generation time and small number of chromosomes (4), has encouraged geneticists to isolate an enormous collection of mutant

flies, that vary from the wild type in a variety of ways. Since the late 1960s, several groups have searched for *Drosophila* mutants with behavioural defects possibly associated with ion-channel mutations. These behavioural defects fall into four major classes: a) abnormal motor control and violent shaking while under ether anaesthesia; this phenotype has been associated with hyperexcitable membrane; b) reversible paralysis at elevated temperatures; this phenotype has been associated with a reduction in membrane excitability; c) reversible paralysis caused by prolonged sensory stimulation (such as vortexing); the neural basis for this phenotype is unclear; and d) cold-sensitive paralysis; the basis for this phenotype is also not known. In addition to screens for the behavioural abnormalities listed above, there have been searches for mutations that enhance or suppress either leg-shaking behaviour or temperature-sensitive paralysis. Interestingly, mutations that enhance leg shaking behaviour, often cause leg shaking by themselves; conversely mutations that suppress leg-shaking often show temperature-dependent paralysis. A similar phenomenon is observed for suppressers and enhancers of temperature-sensitive paralytics (114). A large collection of these behavioural mutants exists although few have been described in detail.

Despite the small size of fruit fly neurons, electrophysiological techniques have been quite gainfully employed in the study of *Drosphila* ion channels. Electrical currents in *Drosophila* muscle and nerve have been quite extensively characterized. Several different tissue preparations from embryo, larva, pupa and adult have been examined by intracellular recordings, and also by voltage-clamp and patch-clamp methods. These analyses have shown that several of the *Drosophila* behavioural mutants have defects in specific ionic currents. A comprehensive account of the different mutants is beyond the scope of this essay; however, most published material on this subject has been reviewed in detail (114, 115, 116).

Thus, a unique combination of genetic and electrophysiological methods are available in *Drosophila*, for the identification of genes that affect channel function. Molecular cloning of these genes is possible without prior biochemical purification of the

the proteins. These experiments may yield not only new ion-channel genes, but also genes whose products serve to regulate channel expression and assembly. The subsequent chapters describe work that interfaces, in a sense, genetic studies on *Drosophila*, and physiological and biochemical studies on vertebrate voltage-gated ion channels.

References

1. D. Nachmansohn, E. Neumann, *Chemical and molecular basis of nerve activity*. (Academic Press Inc., New York, 1975).

2. I. Cooke, M. Lipkin, (Holt, Rinehart and Winston Inc., New York, U.S.A., 1972),

3. A. L. Hodgkin, B. Katz, *J. Physiol.* 108, 33-77 (1949).

4. O. Loewi, E. Navratil, *Pflugers Arch.* 214, 678-688 (1926).

5. H. H. Dale, W. Feldberg, M. Vogt, *J. Physiol.* 86, 353-380 (1936).

6. P. Fatt, B. Katz, *J. Physiol.* 117, 109-128 (1952).

7. J. Del Castillo, B. Katz, *J. Physiol.* 124, 560-573 (1954).

8. B. Katz, R. Miledi, *J. Physiol.* 167, 23-38 (1967).

9. J. Heuser, T. Reese, D. Landis, *J. Neurocytol.* 3, 109-131 (1974).

10. E. J. Furshpan, D. D. Potter, *J. Physiol.* 145, 289-325 (1959).

11. T. Narahashi, J. Moore, W. Scott, *J. Gen. Physiol.* 47, 965-964 (1964).

12. C. Armstrong, L. Binstock, *J. Gen. Physiol.* 48, 859-872 (1965).

13. W. Catterall, *Ann. Rev. Pharmacol. Toxicol.* 20, 15-43 (1980).

14. W. A. Catterall, *Ann. Rev. Biochem.* 55, 953-985 (1986).

15. C. M. Armstrong, F. Benzanilla, *Nature* 242, 459-461 (1973).

16. E. Levitan, *et al. Nature* 335, 76-79 (1988).

17. R. Nicoll, *Science* 241, 545-551 (1988).

18. A. Ashkenazi, J. Ramachandran, D. Capon, *Nature* 340, 146-150 (1989).

19. D. Julius, K. N. Huang, T. J. Livelli, R. Axel, T. M. Jessel, *Proc. Natl. Acad. Sci.* 87, 928-932 (1990).

20. N. Unwin, *Neuron* 3, 665-676 (1989).

21. M. Noda *et al., Nature* 299, 793-797 (1982).

22. M. Mishina *et al., Nature* 307, 604-608 (1984).

23. T. Claudio *et al., Science* 238, 1688-1694 (1987).

24. M. Raftery, M. Hunkapillar, C. Strader, L. Hood, *Science* 208, 1454-1457 (1980).

25. K. Imoto *et al.*, *Nature* 324, 670-674 (1986).

26. R. Stroud, *Neurosci. Commentar.* 1, 124-138 (1983).

27. E. Kubalek, S. Ralston, J. Lindstrom, N. Unwin, *J. Cell. Biol.* 105, 9-18 (1987).

28. M. Mishina *et al.*, *Nature* 313, 364-369 (1985).

29. K. Imoto *et al.*, *Nature* 335, 645-648 (1988).

30. R. Leonard, C. Labarca, P. Charnet, N. Davidson, H. A. Lester, *Science* 242, 1578-1581 (1988).

31. R. Duvosin, E. Deneris, J. Patrick, S. Heinemann, *Neuron* 3, 487-496 (1989).

32. R. L. Papke, J. Boulter, J. Patrick, S. Heinemann, *Neuron* 3, 589-596 (1989).

33. P. Schofield *et al.*, *Nature* 328, 221-227 (1987).

34. G. Grenningloh *et al.*, *Nature* 328, 215-220 (1987).

35. M. Hollmann, O'Shea-Greenfield, S. Rogers, S. Heinemann, *Nature* 342, 643-648 (1989).

36. U. B. Kaupp *et al.*, *Nature* 342, 762-766 (1989).

37. T. Furuichi *et al.*, *Natyre* 342, 32-38 (1989).

38. H. Takeshima *et al.*, *Nature* 339, 439-445 (1989).

39. F. Sigworth, E. Neher, *Nature* 287, 447-449 (1980).

40. R. L. Rosenberg, S. A. Tomiko, W. S. Agnew, *Proc. Natl. Acad. Sci. U.S.A.* 81, 5594-5598 (1984).

41. M. Baer, P. Best, H. Reuter, *Nature* 263, 344-345 (1976).

42. J. Harris, S. Thesleff, *Acta physiol. scand.* 83, 382-388 (1971).

43. P. A. Pappone, *J. Physiol.* 306, 337-410 (1980).

44. L. Cruz *et al.*, *J. Biol. Chem.* 260, 9280-9288 (1985).

45. E. Jaimovich, M. Ildefonse, J. Barnahim, O. Rougier, M. Lazdunsky, *Proc. Natl. Acad. Sci. U.S.A* 79, 3986-3900 (1982).

46. B. Haimovich, D. L. Schotland, W. E. Fieles, R. L. Barchi, *J. Neurosci.* 7, 2957-2966 (1987).

47. B. Rudy, *J. Physiol.* 249, 22-24P (1975).

48. B. Rudy, *J. Physiol.* 312, 531-549 (1981).

49. W. F. Gilly, C. M. Armstrong, *Nature* 309, 448-450 (1984).

50. R. Llinas, M. Sugumori, *J. Phys.* 305, 171-195 (1980).

51. R. E. Weiss, R. Horn, *Science* 233, 361-364 (1986).

52. J. B. Patlak, M. Ortiz, *J. Gen. Physiol.* 86, 89-104 (1985).

53. B. A. Barres, L. L. Y. Chun, D. P. Corey, *Neuron* 2, 1375-1388 (1989).

54. M. Noda *et al.*, *Nature* 312, 121-127 (1984).

55. E. M. Kosower, *FEBS Lett.* 182, 234-242 (1985).

56. R. E. Greenblatt, Y. Blatt, M. Montal, *FEBS Lett.* 193, 125-134 (1985).

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

58. R. H. Guy, 33, 289-308 (1988).

59. M. Noda *et al.*, *Nature* 320, 188-192 (1986).

60. J. Trimmer *et al.*, *Neuron* 3, 33-49 (1989).

61. V. J. Auld *et al.*, *Neuron* 1, 449-461 (1988).

62. M. Noda *et al.*, *Nature* 320, 826-828 (1986).

63. D. Krafte, T. Snutch, J. Leonard, N. Davidson, H. Lester, *J. Neurosci.* 8, 2859-2868 (1988).

64. N. Dascal, *The use of Xenopus oocytes for the study of ion-channels* (CRC Press, 1987).

65. H. Lubbert *et al.*, *Proc. Natl. Acad. Sci.* 84, 4332-4336 (1987).

66. D. Julius, A. MacDermott, R. Axel, T. Jessel, *Science* 241, 558-564 (1988).

67. H. Suzuki *et al.*, 228, 195-200 (1988).

68. V. Auld *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 87, 323-327 (1990).

69. B. Hille, *Ionic channels of excitable membrane* (Sinauer, Sunderland MA, 1984).

70. C. Miller, *Neuron* 2, 1195-1205 (1989).

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

72. W. Stuhmer *et al.*, *Nature* 339, 597-603 (1989).

73. E. Rojas, C. Armstrong, *Nature, New Biol.* 229, 177-178 (1971).

74. E. Rojas, B. Rudy, *J. Physiol.* 262, 501-531. (1976).

75. P. M. Vassilev, T. Scheuer, W. Catterall, *Science* 241, 1658-1661 (1988).

76. H. Meiri *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 84, 5058-5062 (1987).

77. M. Noda, H. Suzuki, S. Numa, W. Stuhmer, *FEBS Letters* 213-216, (1989).

78. S. Oiki, W. Danho, M. Montal, *Proc. Natl. Acad. Sci. U.S.A.* 85, 8703-8707 (1988).

79. M. Tosteson, D. Auld, D. Tosteson, *Proc. Natl. Acad. Sci. U.S.A.* 86, 707-710 (1989).

80. H. A. Lester, *Science* 241, 1057-1063 (1988).

81. B. Rudy, *Neurosci.* 25, 729-749 (1988).

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

83. P. Reinhart, S. Chung, I. Levitan, *Neuron* 2, 1031-1041 (1989).

84. J. Connor, C. Stevens, *J. Physiol.* 213, 21-30 (1971).

85. J. M. Dubois, *J. Physiol.* 318, 297-316 (1981).

86. F. Conti, B. Hille, W. Nonner, *J. Physiol.* 353, 199-230 (1984).

87. G. Harris, L. Henderson, N. Spitzer, *Neuron* 1, 739-750 (1988).

88. R. Coronado, R. Lattore, H. Mautner, *Biophys. J.* 45, 289-299 (1984).

89. Y. Srinivasan, L. Elmer, J. Davis, V. Bennet, K. Angelides, *Nature* 333, 177-180 (1988).

90. E. Moczydlowski, K. Lucchesi, A. Ravindran, *J. Membrane. Biol.* 105, 95-111 (1988).

91. N. B. Standen *et al.*, *Science* 245, 177-180 (1989).

92. E. Carmiliet, *J. Pharmacol. Exp. Ther.* 232, 817-825 (1985).

93. H. Rehm, M. Lazdunski, *Proc. Natl. Acad. Sci. U.S.A.* 85, 4919-4923 (1988).

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

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

96. O. Pongs *et al.*, *EMBO J.* 7, 1087-1096 (1988).

97. K. J. McCormack *et al.*, *Nature* 340, (1989).

98. T. Abel, T. Maniatis, *Nature* 341, 24 (1989).

99. M. Tanouye, A. Ferrus, S. Fujita, *Proc. Natl. Acad. Sci. U.S.A.* 78, 6548-6552 (1981).

100. F. Haugland, C.-F. Wu, *J. Neurosci.* In Press, In Press (1990).

101. L. Timpe, L. Jan, *J. Neurosci.* 7, 1307-1317 (1987).

102. L. C. Timpe *et al.*, *Nature* 331, 143-145 (1988).

103. L. Timpe, Y. Jan, L. Jan, *Neuron* 1, 659-667 (1988).

104. L. E. Iverson, M. A. Tanouye, H. A. Lester, N. Davidson, B. Rudy, *PNAS* 85, 5723-5727 (1988).

105. L. Iverson, B. Rudy, *J. Neurosci.* In Press. (1990).

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

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

108. T. Scheuer *et al.*, *Science* 247, 854-858 (1990).

109. T. Tanabe *et al.*, *Nature* 328, 313-318 (1987).

110. D. M. Papazian, L. C. Timpe, Y. N. Jan, L. Y. Jan, *Soc. Neurosci. Abs.* 15, 337 (1989).

111. R. Mackinnon, C. Miller, *J. Gen. Physiol.* 91, 335-349 (1988).

112. R. MacKinnon, C. Miller, *Science* 245, 1382-1385 (1989).

113. K. McCormack *et al.*, *In Preparation* (1990).

114. B. Ganetzky, C. F. Wu, *Ann. Rev. Genet.* 20, 3-44 (1986).

115. M. A. Tanouye, C. A. Kamb, L. E. Iverson, L. Salkoff, *Ann. Rev. Neurosci.* 9, 225-276 (1986).

116. S. Singh, C. F. Wu, *Neuron* 2, 1325-1329 (1989).

117. O. Belluzzi, O. Sacchi, E. Wanke, *J. Physiol.* 358, 91-108 (1985).

118. O. Belluzzi, O. Sacchi, E. Wanke, *J. Physiol.* 358, 109-130 (1985).

119. M. Galvan, C. Sedlmeir, *J. Physiol.* 356, 115-133 (1984).

120. M. Segal, J. L. Barker, *J. Neurophysiol.* 51, 1409-1433 (1984).

121. A. Duval, C. Leoty, *J. Physiol.* 307, 43-57 (1980).

122. S. Hagiwara, K. Kawa, *J. Physiol.* 356, 135-149 (1984).

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

124. I. R. Josephson, J. Sanchez-Chapula, A. M. Brown, *Circulation Res.* 54, 157-162 (1984).

125. J. Williams, R. North, S. Shefner, S. Nishi, T. Egan, *Neuroscience* 13, 137-156 (1984).

126. E. Cooper, A. Schrier, *J. Physiol.* 369, 199-208 (1985).

127. J. Halliwell, L. Othman, A. Pelchen-Matthews, J. Dolly, *Proc. Natl. Acad. Sci. U.S.A.* 83, 493-497 (1986).

128. C. Stansfield, S. Marsh, J. Halliwell, D. Brown, *Neurosci. Lett.* 64, 209-304 (1986).

129. T. Hoshi, R. Aldrich, *J. Gen. Physiol.* 91, 107-131 (1988).

130. T. Hoshi, W. N. Zagotta, R. W. Aldrich, (1989).

Table 1. Free ionic concentrations and equilibrium potentials for mammalian skeletal muscle.

Ion	Extracellular Concentration (mM)	Intracellular Concentration (mM)	$\frac{[Ion]_o}{[Ion]_i}$	Equilibrium Potential (mV)
Na^+	145	12	12	+67
K^+	4	155	0.026	-98
Ca^{2+}	1.5	$<10^{-7}M$	>15000	+128
Cl^-	123	4.2	30	-90

Hille, 1984.

Table 2. Properties of purified sodium-channel preparations.

Source	Oligomeric molecular mass (kd)	Specific saxitoxin binding (mol/mol)	Subunit molecular masses: Native (kd)	Subunit molecular masses: Deglycosylated (kd)
Eel electroplax	260	0.25-0.35	260	208
Mammalian brain	316	0.9	α: 260 ß1: 36 ß2: 33	200-220 23 21
Mammalian skeletal muscle	314	0.85	α: 260 ß: 38	

Catterall, 1986

Table 3. Voltage-gated potassium channels in rat.

Preparation	Activation: Threshold (mV)	Activation: kinetics (ms)	Inactivation rate/$k_{0.5}$ (ms/mV)	4AP block	TEA block external	Others	Other properties: Reference
Sympathetic neurons	1) >-40 (25°C) 2) -60	10-20 (26mv, 25°C) v. fast	~5000 55/-75	NB Kd> 1mM	3mM		(117, 118, 119)
Hippocampal pyramidal neurons	~-40	~50 (20°C)	2000-4000 (20°C)	NB 5mM	K_d~10mM		(120)
Skeletal muscle 20°C	~-50	1) 6 2) 60	200-300 1500-3000	Block NB	Block 40mM Block>>40mM		(121) Slow I in slow-twitch muscle
Spermatogenic cells	~-40	~20 (22°C; -10mV)			Block 126mM		(122)
Cultured hippo-campal neuron	-60	3-10	10-40/-75	Kd~2mM			(120, 123)
Cardiac ventricular cells	-40	2-10	20-40/-60	Kd~2mM			(124)
Locus coeruleus neurons	-60	-	~100/-50(100%)	Kd~1mM			(125)
Cultured sensory neurons	-50	-	30-50/<-65	-			(126)
CA1 hippocampal cells	-60	v. fast	50/,-60	-		1mM DTX	(127)
Sensory neurons nodose ganglion	-60	-	1000-2000	Block 30μM		DTX	(128)
PC12 cells	Kx) 0 Kw) -20 Ky) -60 Kz) -55	50 10 (0mV) variable 2.5 (10mV)	v. slow/-120 40-80/-70 v. slow 100-500/-70	No Yes No Yes (?)	Block Yes Yes Yes		(129, 130) g = 5-9pS " g = 14-18pS " g = 5-9pS " g = 11-14pS

Chapter 2

Two Sodium-Channel Genes in *Drosophila*: Implications for Channel Diversity.

Mani Ramaswami and Mark A. Tanouye

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Abstract

We describe two *Drosophila melanogaster* transcription units that are highly homologous to a rat Na^+-channel cDNA. They appear to encode the major subunits of two distinct Na^+-channel proteins. One of these maps to the second chromosome and is identical to a Na^+-channel gene whose partial sequence has been previously reported (Salkoff et al., 1987, *Science* 237, 744-749). The other transcription unit maps to position 14C/D, on the X chromosome, close to the paralyzed (*para*) gene. Mutations in *para* affect membrane excitability in *Drosophila* neurons (Ganetzky and Wu, 1986, *Annu. Rev. Genet.* 20, 30-34). Sequence comparisons suggest that two Na^+-channel genes arose early in evolution, before the divergence of vertebrate and invertebrate lines.

Introduction

Ion channels are a diverse group of integral membrane proteins regulating the passage of ions through cell membranes. Electrophysiological, pharmacological and biochemical methods have distinguished several types of sodium and calcium channels, generally involved in the depolarization of excitable membranes (1-3). An even larger number of distinct potassium channel types, involved in membrane repolarization, has also been detected (4). The differential distribution of these channel types underlies the range of electrical responses in neurons (5).

The cloning of ion channel genes allows the detailed biochemical and biophysical characterization of channel proteins. Recent results have shown that voltage-gated channels share several sequence motifs (2, 6-15). Na^+ channels contain four internal repeats about 250 residues in size that are approximately 50% identical to one another. These repeats are also called homology domains. In each domain multiple hydrophobic segments called S1, S2, S3, S5 and S6 flank a positively charged segment called S4. S4 segments consist of 4-8 iterations of a three residue sequence Arg-X-X, where X is a hydrophobic residue and Lys is sometimes substituted for Arg. A putative Ca^{2+} channel has an identical structural

organization (15). All K^+ channels cloned so far are similar in structure to a single homology domain of the Na^+ channel. The data suggest that these different channel types may have evolved from a single ancestral voltage-gated channel that arose early in phylogeny.

The isolation of ion channel genes in an organism like *Drosophila*, where genetic manipulations are feasible, facilitates the identification of other genes involved in ion channel function. Several mutations affecting neural excitability have been identified in *Drosophila* (16, 17). Mutations that affect specific classes of K^+ channels (*Sh*, *eag*, *slo* and *Hk*) cause abnormalities associated with increased membrane excitability (17). Conversely, mutations that affect Na^+ currents ($para^{ts}$, nap^{ts} and $tip\text{-}E^{ts}$) cause an overall decrease in membrane excitability (16-19). These mutations might identify structural genes for ion channels, or genes for proteins involved in the synthesis, membrane distribution or modulation of ion channels.

We have searched *D. melanogaster* genomic libraries for sequences similar to an mRNA for the large subunit of a voltage-gated Na^+ channel from rat brain, with goals to: a) complement genetic approaches; b) analyze the diversity of Na^+-channel genes; and c) further understand the structure and evolution of ion channels. In this paper, we report the partial characterization of two transcription units that appear to encode distinct Na^+-channel proteins. One of these genes probably corresponds to the *para* locus.

Materials and Methods

(a) Standard Techniques:

Standard methods of molecular biology were as described in laboratory manuals (20, 21). Radiolabelled DNA probes were synthesized using the random hexamer primer method (22). *Drosophila* genomic DNA preparations, *in situ* hybridizations to polytene chromosomes and DNA sequence analysis were performed as described in (9, 10, 23-25).

(b) Isolation of *Drosophila* genomic DNA clones homologous to a rat Na^+-channel gene:

Three cDNA clones encoding parts of the major subunit of the rat brain Na^+ channel, RatIIA (Fig. 1, Ref. 7), were used as hybridization probes to screen lambda phage libraries of *Drosophila* genomic DNA. The libraries were constructed by Dr. C.A. Kamb (9). Rat Na^+-channel cDNAs were a gift from Drs. A. Goldin and N. Davidson at Caltech and V. Auld and R. Dunn of the University of Toronto. The rat cDNA clones AG141, NA2.2, and NA8.4 contained coding sequences for approximately 75% of RatIIA (Fig. 1, Ref. 7). The sequences most highly conserved between different Na^+ channels lie largely within the homology domains (2, 7, 8). NA2.2 contains sequences encoding domain A; and NA8.4 encodes domains C and D. AG141 contains less conserved sequences. The hybridizations were carried out at 25° C in 0.75M NaCl, 0.025M Na_2HPO_4, 0.001M EDTA, 0.1% $NaDodSO_4$, 0.05M Tris, 100 ug/ml denatured salmon sperm DNA, 50% formamide and 10% dextran sulfate at pH 7.5. The filters were rinsed at room temperature in 0.3M NaCl, 0.03M sodium citrate and 0.1% $NaDodSO_4$, and washed for 30 minutes at 55° C in the same solution. No cross-hybridization was observed when the wash temperature was raised to 65° C. Forty-two recombinant phage clones were isolated. These were placed into 21 groups of non-overlapping clones based on a comparison of their restriction maps, and on hybridization experiments in which DNA from all 42 clones was probed with restriction fragments purified from particular clones. In some cases the assignments were verified by *in situ* hybridizations of cloned DNA to polytene chromosomes from larval salivary glands. The groups were named for a representative clone.

In a second screen, 5 of the 21 groups were identified as strong candidates for ion channel genes. About 70% of single-copy DNA from *Drosophila melanogaster* does not form stable hybrids with DNA from a distant species of *Drosophila, D. virilis*, under conditions corresponding to approximately 60% homology (26). Only 5 groups (A20,

A4.3, A3.13, A4.5 and A4.11) had specific restriction fragments (1.5 kb to 3.5 kb in size) that cross-hybridized with both rat Na^+-channel cDNAs and *D. virilis* genomic DNA. Hence only these contained DNA sequences similar to the rat Na^+-channel gene that were also phylogenetically conserved. Two groups (A4.3 and A3.13) that cross-hybridized most strongly with the rat Na^+-channel cDNAs were chosen for further characterization. Both these groups were initially isolated with the rat probe NA8.4 (Fig. 1). The order of restriction endonuclease cleavage sites in the cloned DNA was determined (Fig. 2). Restriction fragments that cross-hybridized with rat channel sequences were used to screen *Drosophila* cDNA libraries.

(c) Screening cDNA libraries:

Complementary DNA clones from A4.3 and A3.13 were obtained from two adult head cDNA libraries (gifts from Dr. P. Salvaterra and Dr. C. Zuker). In all, 14 independent clones representing A4.3 and 10 representing A3.13 were identified. The cDNAs were subcloned into plasmid vectors (Bluescript and mp18) for further analysis.

Results and Discussion

A4.3 defines a *Drosophila* Na^+ channel:

The partial nucleotide sequence of a cDNA (B1) from A4.3 was determined. A comparison (data not shown) shows that B1 is about 59% identical in nucleotide sequence to a region of the rat Na^+-channel gene, RSC2 (2). This region encodes segments S4, S5 and a portion of S3 from homology domain C of the rat channel (amino acid residues 1287 to 1364, Ref. 2). The amino acid sequence deduced from B1 is identically contained in DSC, a putative *Drosophila* Na^+ channel whose partial sequence has been reported recently (amino acid residues 1080 to 1155, Ref. 8). The nucleotide sequences of B1 and DSC are identical over approximately 240 nucleotides (27). It has not been clearly demonstrated that

DSC encodes a functional sodium-channel protein. However the sequence data strongly support this hypothesis (8).

The genomic clone A4.3 was mapped *in situ* on salivary gland chromosomes to a single site at 60E (Fig. 4a). This location is identical to the reported cytogenetic map position of the DSC gene (8). Thus it appears that A4.3 and DSC define the same *Drosophila* gene. This conclusion is based on (a) the sequence identity we find between the two genes; (b) the identical cytological map positions of A4.3 and DSC; and (c) unique hybridization signals obtained with probes from A4.3 on blots of genomic DNA.

A3.13 defines a second *Drosophila* Na^+-channel gene:

The order of restriction enzyme cleavage sites in genomic DNA around A3.13 is shown (Fig. 2b). Three non-contiguous restriction fragments in this DNA cross-hybridize with rat Na^+-channel cDNAs. Thus, at this level of analysis, it appears that homology to the rat Na^+-channel gene spans several kilobases of genomic DNA. We determined the nucleotide sequence of a cDNA (P15) from A3.13. The sequence (660 base pairs) is 68% identical to a region of RSC2 (2) which encodes homology domain D. A single open reading frame extends through the entire sequence of P15. The deduced amino acid sequence shows structural motifs characteristic of voltage-gated ion channels: an S4-like segment is flanked by three hydrophobic stretches of amino acid residues (Fig. 3). A majority of nucleotide differences between P15 and RSC2 are in "wobble base" positions and do not change the predicted polypeptides. Figure 3 shows aligned amino acid sequences deduced from P15, RSC2 and DSC. A total of 141 residues are identical between rat and P15 sequences over the 212 residues shown; while 108 residues are identical between P15 and DSC. The S4-like segment of all 3 sequences contain exactly 8 positively charged residues; the hydrophobic segments are also highly conserved. Taken together, the data strongly suggest that A3.13 encodes the major subunit of a *Drosophila* Na^+ channel.

A3.13 probably corresponds to the *para* locus of *Drosophila*:

In situ hybridizations to *Drosophila* polytene chromosomes show that the cytological location of A3.13 is 14C/D (Fig. 4b), virtually identical to the *para* locus that has been mapped to 14C6-8 (17). A chromosomal walk through *para* by Loughney and Ganetzky has included many chromosomal breakpoints that uncover *para* mutations and sequences of para cDNAs reveal homology to sodium-channel genes (34, 35). DNA from A3.13 hybridizes to cloned DNA from this walk (Loughney and Ganetzky, personal communication). Thus, a comparison of results from the two laboratories indicates that A3.13 derives from the *para* locus.

Many lines of evidence have previously suggested that *para* could be a structural gene for a Na^+ channel: a) several *para* alleles show rapid and reversible paralysis above characteristic restrictive temperatures (17, 28); b) paralysis is associated with a temperature-dependent block in action potential propagation in some neurons (17, 29, 30, 31); c) cultured neurons from $para^{ts}$ larvae show a temperature-dependent resistance to veratridine, a neurotoxin that binds and stabilizes Na^+ channels in an "open" conformation (32); and d) electrophysiological recordings from cultured neurons from *para* embryos show a significant reduction in the number of neurons expressing a Na^+ current (33). Taken together, these lines of evidence strongly suggest that *para* encodes the major subunit of a *Drosophila* Na^+ channel.

Genes for two neural excitability mutants in *Drosophila* (*para* and *Shaker*), that were implicated in ion channel function, have now been cloned (9, 11, 13). Both appear to encode ion channel proteins. This increases the likelihood that behavioral mutants with similar phenotypes identify additional ion channel genes. These findings also increase interest in the molecular characterization of loci that genetically interact with *Shaker* and *para* (16, 17).

Implications for Na^+ channel evolution and diversity:

There is increasing evidence that vertebrate Na^+ channels constitute a diverse family of proteins (1). Electrophysiological and pharmacological methods have distinguished two classes of Na^+ current in rat skeletal muscle membranes, and a slightly different current in rat brain (1). Distinctive Na^+ currents have been seen in Purkinje cells of guinea pigs (36) and in type I astrocytes in rat optic nerve (37). Na^+ channels in rat muscle membrane are different from those in T-tubular membranes (38, 39). In part this diversity may be due to the existence of multiple genes, as mRNAs for three distinct Na^+ channels are simultaneously expressed in rat brain (2, 7). The three proteins encoded by these transcripts are 95% identical to each other over the region depicted in figure 3. Table 1 shows sequence homologies between four putative Na^+-channel proteins. These figures have been computed over about 210 residues for which sequence is available for all the channels. The predicted protein sequence of *para* is 67% identical to the rat channel (RSC2) and 62% identical to the eel channel. It is, however, only 51% identical to DSC over the same sequence. The DSC sequence is 54% and 56% identical to the rat and eel channels respectively. All four Na^+-channel proteins are about 30% identical to the dihydropyridine receptor that has been proposed to function as a voltage-gated Ca^{2+} channel (15). The striking sequence similarity among these genes, suggests that they share a common evolutionary origin. Voltage-gated Ca^{2+} channels have been detected in protists, while voltage-gated Na^+ channels appear to have evolved more recently (5). Thus, the sequence data are consistent with an evolutionary scenario in which the first ancestral Na^+ channel arose by duplication of, and divergence from, a voltage-gated Ca^{2+} channel. The strong homology among the Na^+-channel genes in vertebrates and in *Drosophila*, suggests that voltage-gated Na^+ channels evolved before the divergence of vertebrates and invertebrates about 600 million years ago (8). The fact that *para* is more closely related to the known vertebrate channels than it is to DSC is easily explained if DSC and *para* diverged from each other before *para* diverged from the vertebrate Na^+ channels. Though our arguments

are based on limited sequence information, it appears that two distinct Na^+-channel genes existed in the early Cambrian period, even before the divergence of vertebrates from invertebrates. On this basis one would predict that DSC might define a subfamily of vertebrate Na^+ channels that are yet to be molecularly identified.

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References

1. Barchi, R.L. (1987) *TINS*. 10, 221-223.

2. Noda,M., Ikeda, T., Kayano, T., Suzuki, H., Takeshima, H., Kurasaki, M., Takahashi, H. & Numa, S. (1986) *Nature (London)* 320, 188-192.

3. Catterall, W.A. (1986) *Ann. Rev. Biochem.* 55, 953-985.

4. Rudy, B. (1988) *Neuroscience*, 25, 729-749.

5. Hille, B. (1984) *Ionic channels of excitable membranes* (Sunderland, Ma: Sinauer).

6. Noda, M., Shimizu, S., Tanabe, T., Takai, T., Kayano, T., Ikeda, T., Takahashi, H., Nakayama, Y., Kanaoka, Y., Minamino, N., Kangawa, K., Matsuo, H., Raftery, M.A., Hirose, T., Inayama, S., Hayashida, H., Miyata, T. & Numa, S. (1984) *Nature (London)* 312, 121-127.

7. Auld, V.J., Goldin, A., Krafte, D., Marshall, J., Dunn, J.M., Catterall, W.A., Lester, H.A., Davidson, N. & Dunn, R.J. (1988) *Neuron*, 1, 449-461.

8. Salkoff, L., Butler, A., Wei, A., Scavarda, N., Giffen, K., Ifune, K., Goodman, R. & Mandel, G. (1987) *Science* 237, 744-749.

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

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

11. Papazian, D.M., Schwarz, T.L., Tempel., Jan, Y.N. & Jan, L.Y. (1987) *Science* 237, 749-753.

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

13. Baumann, A., Krah-Jentgens, I., Mueller, R., Mueller-Holtkamp, Seidel, R., Kecskemethy,N., Ferrus, A. & Pongs, O. (1987) *EMBO J.* 6, 3419-3429.

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

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

16. Tanouye, M.A., Kamb, C.A., Iverson, L.E., & Salkoff, L. (1986) *Ann. Rev. Neurosci.* 9, 225-276.

17. Ganetzky, B. & Wu, C.F. (1986) *Ann. Rev. Genet.* 20, 13-44.

18. Ganetzky, B. (1984) *Genetics* 108, 897-911.

19. Ganetzky, B.(1986) *J. Neurogenetics* 3, 19-31.

20. Maniatis, T., Fritsch, E.F., & Sambrook, J. (1982) *Molecular cloning: A Laboratory Manual* (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory).

21. Davis, R.W., Botstein, D. & Roth, J.R. (1980) *Advanced bacterial genetics. A manual for genetic engineering* (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory).

22. Feinberg, A.P. & Vogelstein, B. (1983) *Anal. Biochem.* 132, 6-13.

23. Pardue, M., & Gall, J. (1975) in *Methods in Cell Biology* 10, ed. D. Prescott (Academic Press, New York), 1-17.

24. Sanger, F., Niklen, S. & Coulson, A.R., (1977) *Proc Natl. Acad. Sci. USA*. 84, 5463-5467.

25. Messing, J. (1983) *Methods Enzymol.* 101, 20.

26. Zwiebel, L.J., Cohn, V.H., Wright, D.R. & Moore, G.P. (1982) *J. Mol. Evol.* 19, 62-71.

27. Salkoff, L., Butler, A., Scavarda. N. & Wei, A. (1987) *Nucleic Acids Res.* 15, 8569-8573.

28. Suzuki, D.T., Grigliatti,T. & Williamson, R. (1971) *Proc. Natl. Acad. Sci.* 68, 890-893.

29. Siddiqi,O. & Benzer,S. (1976) *Proc. Natl. Acad. Sci.* 73, 3253-3257.

30. Benshalom, G. & Dagan, D. (1981) *J. Comp. Phys.* 144, 409-417.

31. Wu, C.F. & Ganetzky, B. (1980) *Nature (London)* 286, 814-816.

32. Suzuki, N. & Wu, C.F. (1984) *J. Neurogenet.* 1, 225-238.

33. O'Dowd, D., Germeraad, S. & Aldrich, R. (1987) *Soc. Neuroscience Abs.* 13, 577.

34. Ganetzky, B., Loughney, K. & Wu, C.F. (1986) in *Tetrodotoxin, Saxitoxin and the Molecular Biology of the Sodium Channel*, eds. Kao, C.Y & Levinson, S.R. (NY. Acad. Science, New York), pp. 325-337.

35. Ganetzky, B. & Loughney, K. (1988) *Soc. Neurosci. Abs.*, 14, 598.

36. Llinas, R. & Sugumori, M. (1980) *J. Phys.* 305, 171-195.

37. Barres, B.A., Chun, L.L.Y. & Corey, D.P. (1987) *Soc. Neurosci. Abs.* 13, 577.

38. Jaimovich, E., Ildefonse, M., Barnahim, J., Rougier, O. & Lazdunsky, M. (1982) *Proc. Natl. Acad. Sci.* 79, 3986-3900.

39. Haimovich,B., Schotland, D.L., Fieles, W.E. & Barchi, R.L. (1987) *J. Neurosci.* 7, 2957-2966.

Figure 1: Representations of the rat Na^+ channel RatIIA, and the cDNA clones used as hybridization probes.

The major subunit of RatIIA is a 2000 amino acid polypeptide with four homology domains. In the figure the domains are labelled as A,B,C, and D from amino to carboxyl terminus. The domains are presumed to assemble in the membrane as "pseudo subunits" around an ion selective pore. The cDNA probes used in the experiments reported here are shown at their apposite positions on the channel gene. NA2.2 is a cDNA extending from nucleotide -10 to 1511 (7). NA8.4 is a cDNA extending from nucleotide 3361 to 5868. AG141 is a 558 b.p. cDNA beginning at nucleotide number 980.

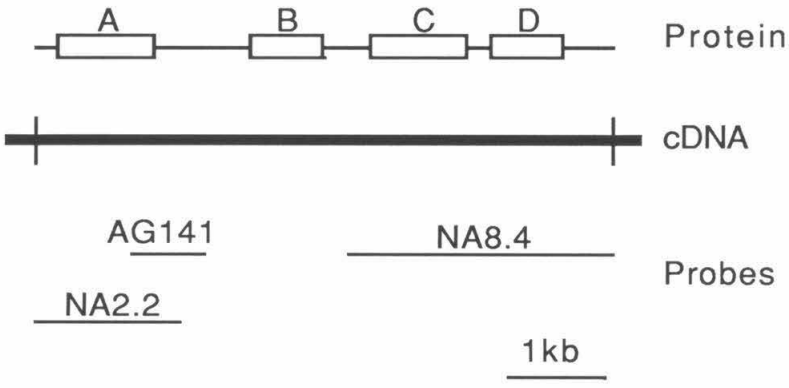
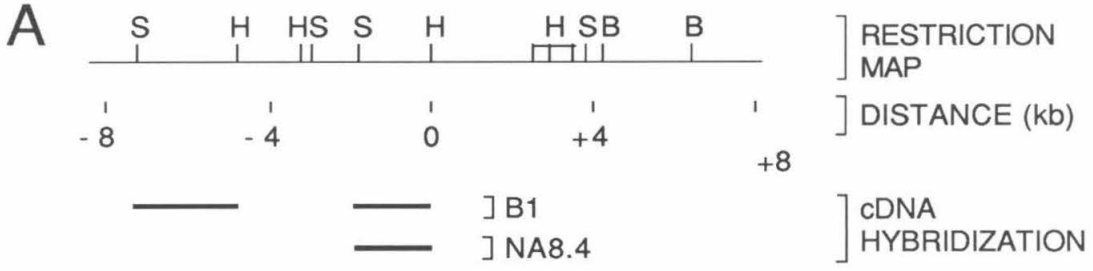

A
B
C
D
Protein
cDNA
AG141
NA8.4
Probes
NA2.2
1kb

Figure 2: Restriction maps of two genomic loci that appear to encode different *Drosophila* Na^+ channels.

A. Map of restriction enzyme cleavage sites in the genomic clone A4.3. The rat cDNA NA8.4 hybridizes to a 1.8 kb SalI HindIII fragment centered at about map position -1. The fragment was used as a hybridization probe to screen *D. melanogaster* cDNA libraries. The other rat cDNAs NA2.2 and AG141 do not cross-hybridize to the DNA shown in the figure. The hybridization pattern of the cDNA clone, B1 is also indicated. The restriction map depicted here is similar to a portion of the DSC map (8) but there are some differences. It is possible that some restriction site polymorphisms exist between the two clones (Salkoff, personal communication). Distance is shown in kilobases of DNA. R is an EcoRI cleavage site; S is a SalI site; B is a BamHI site; and H is a HindIII site.

B. Map of restriction enzyme cleavage sites derived from eight genomic clones of the A3.13 group that were isolated using the rat cDNA probe NA8.4; 3 representative clones (A4.19, A3.13 and A2.39) are shown. Indicated are three non-contiguous restriction fragments that cross-hybridize with NA8.4 and with *D. virilis* genomic DNA. Each of these restriction fragments was used as a hybridization probe to screen *D. melanogaster* cDNA libraries. The hybridization pattern of cDNA P15 described in the text is also shown. The orientation of the gene based on sequence data and mapping of restriction enzyme cleavage sites is, 5' to 3', from left to right.


A
S H HS S H H SB B
RESTRICTION MAP
- 8 - 4 0 +4 +8
DISTANCE (kb)
B1
NA8.4
cDNA HYBRIDIZATION

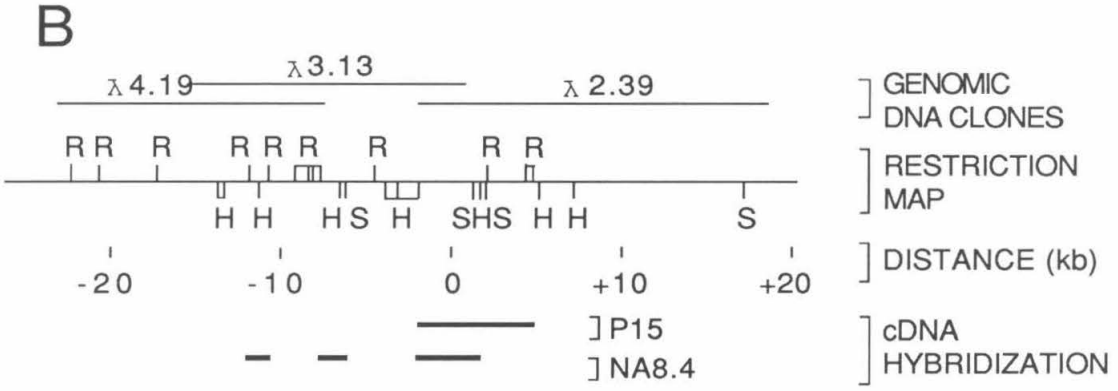

B
λ4.19
λ3.13
λ 2.39
GENOMIC DNA CLONES
R R R R R R R R R
H H H S H S H S H H S
RESTRICTION MAP
-20 -10 0 +10 +20
DISTANCE (kb)
P15
NA8.4
cDNA HYBRIDIZATION

Figure 3: Amino acid sequence derived from A3.13.

The protein sequence deduced from the *Drosophila* cDNA P15 is depicted in the figure. The cDNA is incomplete as judged by the presence of a single unbroken open reading frame that extends through its entire length. For comparison, it is aligned with homologous sequences encoded in the rat Na^+ channel RSC2 (middle) and in a putative Drosophila Na^+ channel DSC (bottom). Amino acid identities with P15 are indicated by dashes. Gaps are inserted for optimal alignment. Segments S3 to S6 of homology domain D of RSC2 are shown in boxes. For reference, the sequences shown in the figure are from amino acid 1596 to 1812 of RSC2 (2) and 1383 to 1595 of DSC (8). The rat Na^+ channel RatIIA (7) is more than 99% identical to RSC2 (2). Nucleotide sequences are available on request.


S3d
S4d
P15 NLFDVVVVVLSILGLVLSDIIEKYFVSPTLLRVVRVAKVGRVLRLVKGAKGIRTLLFALAMSLPALFNICLLLFL
RSC2 -I--F---I---V-MF-AEL-----------F--I-L-RI--I---I-------------M---------G-----
DSC -SV-FLL-LA--F-ILME--MIDLPI-----------FRI--I---IRA-R---R-----VV--------GA--G-
S5d
VMFIFAIFGMSFFMHVKEKSGINDVYNFKTFGQSMILLFQMSTSAGWDGVLDAIINEEACDPPDSD KGYPGN
----Y------N-AY--REV--D-MF--E---N---C---IT-------L-AP-L-SG ---C-PE-DH--SS
IT--Y--L---L-GN-RLQGALD-MV--Q---R--Q----LM-----ND--ESLMIQ ---C- PFIH
S6d
CG S ATVGITFLLSYLVISFLIVINMYIAVILENYSQATEDVQEGLTDDDYDMYYEIWQQFDPEGTQ
VKGD-- N PS---F-FV--II----V-V---------F-V---ESA-P-SE--FE-F--V-EK---DA--
GHTN-N-GNPLLA--YFT-FII--YM---------I-----FN--HQEEEI-IVE--LE-F-IR-SKY--HA--

Figure 4: Cytological mapping of *Drosophila* genomic clones by *in situ* hybridizations to polytene chromosomes.

A. Clone A4.3 maps to 60E at the tip of the right arm of chromosome 2, very close to the reported cytogenetic location of DSC. We are unaware of any neural excitability mutant in *Drosophila* that maps to this location.

The genomic clone A3.13 maps to the region between 14C and 14D on the X chromosome. Chromosome rearrangements that uncover mutations in *para* have been mapped to 14C6-8 (17; see text).

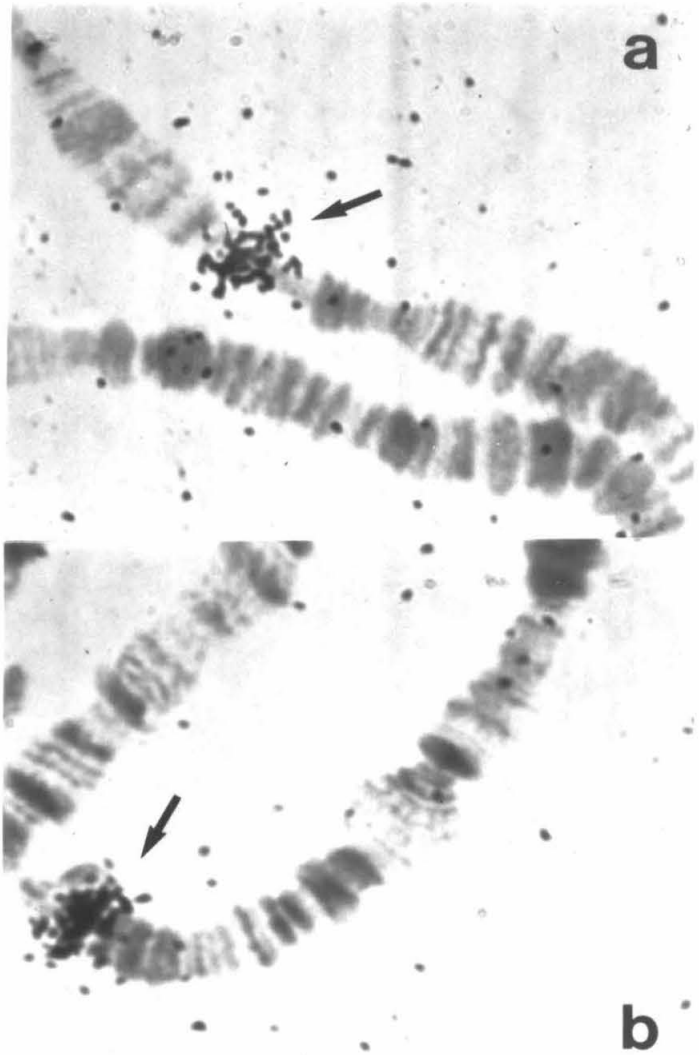

a
b

Table 1: Percent homologies between ion channels.

The homologies have been computed over about 210 amino acids for which sequence information is available for *para*. RSC2 represents a Na^+ channel from rat brain (2); EELSCH represents the Na^+ channel from electric eel (6); DHPR represents the dihydropyridine receptor from rabbit skeletal muscle that is postulated to be a voltage-gated Ca^{2+} channel (15); and DSC represents a putative *Drosophila* Na^+ channel (8). Identical amino acids are scored as 1 and all substitutions are scored as 0. An insertion or deletion is scored as a single substitution independent of its size. We believe it is unlikely that the results of our limited sequence comparisons will be drastically changed when more sequence from *para* becomes available. This is based on the observation that the relative homologies among RSC2, EELSCH and DSC remain fairly constant in all four homology domains of the channel proteins (8). For reference, the sequences are from amino acid 1386 to 1602 for EELSCH (6) and 1183 to 1415 for DHPR (15). The sequences for DSC and RSC2 are as in Fig. 3.

Table 1: Percent homologies between ion channels

	para	DSC	RSC2	EELSCH	DHPR
para	-	51	67	62	30
DSC		-	54	56	29
RSC2			-	75	31
EELSCH				-	29

Chapter 3

Human Potassium-Channel Genes: Molecular Cloning and Functional Expression.

Mani Ramaswami, Medha Gautam, Alexander Kamb, Bernardo Rudy, Mark Tanouye and Mathew Mathew.

(Submitted to *Science*.)

Abstract

Several potassium channel genes present in humans are closely related to the *Shaker* gene of *Drosophila*. Members of the human gene family have specific homologs in rodents suggesting that the individual members arose prior to the mammalian radiation; homologous K^+ channel sequences are more highly conserved than cytochrome C. HuKI, huKII and huKIV channels expressed in *Xenopus* oocytes vary in voltage dependence, kinetics, and sensitivity to pharmacological blockers of potassium channels. Some differences are observed between the pharmacological sensitivities of human channels and the reported sensitivities of their rat homologs.

Introduction

Potassium channels (K^+ channels) are the most diverse class of ion channel with many subtypes usually present in the same cell (*1*). They contribute substantially to diverse nerve action-potential waveforms and bursting frequencies, and to other vital physiological functions such as cardiac pace making (*2*). K^+-channel modulation by second messenger systems has been shown to have profound effects on the function of multineuronal circuits (*3*). In addition, they are targets of many drugs used clinically; for eg. in the treatment of cardiovascular disorders (*4*). For all of these reasons, K^+ channels have been intensely studied by electrophysiologists, molecular biologists and clinical pharmacologists.

The first K^+-channel gene cloned was the *Shaker* gene of *Drosophila* (*5*, *6*). The cloning of *Shaker* has allowed several questions about K^+-channel structure and diversity to be addressed, both in *Drosophila* and in mammals. Mammalian genes related to *Shaker* have recently been identified in mouse and rat by low stringency hybridizations with *Shaker* probes (*7-11*) and by expression cloning methods (*12*). Most of these have been functionally expressed in *Xenopus* oocytes. While additional K^+-channel sequences continue to be identified, molecular and electrophysiological methods are being used by

several groups to analyse structure, activity and physiological functions of cloned K^+ channels.

Studies on human potassium channel genes not only address questions of channel structure and diversity, but also serve additional purposes. As humans diverged from rodents about 80 million years ago, the sequences of human channels may allow one to understand the evolution of channel diversity in higher vertebrates. Functional expression of human potassium channels allows their physiological activities to be directly examined, and to be compared with mouse and rat channels. Understanding interspecific variations in channel sequence and activities, is extremely important if one wishes to extrapolate to humans, the results and conclusions of studies on other animal models. The functional expression of human channels also allows their pharmacological properties to be directly examined; this may be useful in designing and testing potassium channel blockers of potential therapeutic value. In addition, it is possible that some heritable neuromuscular disorders in humans result from defects in K^+ channel genes. For such defects to be identified, and for the evaluation of potential mouse models of the disorders, it is critical to directly examine human K^+ channels.

We report here, the cloning of six voltage-gated K^+ channel genes in humans. We describe the deduced amino acid sequences and functional properties in *Xenopus* oocytes of three of these channel proteins. We discuss similarities and differences among the human channels, and compare them to K^+ channels described in other species.

Results and Discussion

Cloning cDNA from human potassium channel genes:

Six different sequences showing similarity to K^+ channels (huKI to huKVI) were identified using a modified polymerase chain reaction method (*13*). The amplified sequences were used as probes to screen cDNA libraries from human spinal cord, brain stem and basal ganglia (*14*). cDNA clones were identified with five (huKI - huKV) of the

six probes. Their sequences showed considerable homology to fly, rat and mouse K^+ channels, that extended well beyond the amplified region. These sequences were used as probes to isolate overlapping cDNAs from several libraries. All clones identified with the same probe were mapped with respect to one another, and selected clones were sequenced.

HuKIII and huKV cDNA clones have incomplete coding sequences of about 0.7 and 1.0 kilobases, respectively. Even from these incomplete sequences, however, it is clear that they encode distinct K^+-channel proteins. Interestingly, two clones isolated with huKIII and huKV probes contain insertions whose sequences show no similarity to any known K^+ channels or bacterial transposons. The insertions are missing in other cDNAs isolated with the same probe (data not shown). This suggests that the insertions are intervening sequences that are usually removed during RNA splicing and that these cDNA clones resulted from priming of incompletely processed pre-mRNA during library construction. Homologous K^+-channel genes from mouse are reported to have no introns in their coding sequences (*15*).

The cDNA clones isolated with huKI and huKIV probes contain complete coding sequences of two distinct K^+ channel proteins that are 495 and 499 amino acid residues respectively. The cDNA from huKII encodes yet another K^+ channel of at least 603 residues. This cDNA probably lacks about 150 bases of 5' coding sequence, later identified from flanking genomic DNA (Fig. 1; Fig. 2b).

Primary structure of human potassium channel proteins:

The three human K^+ channels (huKI, huKII and huKIV) are similar in overall structure to the *Drosophila Shaker* channels. They contain six hydrophobic segments (H1-H6); between H3 and H4 is a positively charged segment called S4. These distinctive features are conserved in other related K^+ channels and help define a "core" region beginning about 170 residues before H1 and extending through all the hydrophobic segments. In the core region huKI and huKII share 78% aminoacid identity, huKI and

huKIV 86% identity, and huKIV and huKII 79% identity. Most of the variation that occurs here is in the "linkers" between putative transmembrane segments, most significantly between H1 and H2. The high conservation of the core region is similar to that seen in the rodent K^+ channel family (*7-11*). It suggests that the core region is involved in critical channel functions such as voltage-dependent gating, K^+ selectivity and ion permeation.

The three human potassium channels all belong to a closely-related group of K^+ channels, the "*Shaker* class," that have a number of additional features in common. These include consensus sites for post-translational modifications within the core region. A potential phosphorylation site for calcium-calmodulin dependent (CaM II) protein kinases is present in the "core" region of all the human proteins and in other *Shaker*-class channels (Fig 1). This site occurs about 120 amino acids residues prior to H1, in a region that is prbably intracellular. Between H1 and H2, a single consensus site for N-linked glycosylation is present in the three human sequences as in other *Shaker*-class channels (*5*, *7-11*). All models for transmembrane topology of K^+ channels place this site on the extracellular surface.

Human K^+ channels vary considerably outside the core region. There are, however, a few similarities. All three polypeptides end with the sequence Thr-Asp-Val. This sequence is present at the carboxy-terminus of almost all *Shaker*-class channels though its significance is as yet unclear (*5*, *7-11*) (Fig. 2). The three human proteins each contain a cAMP dependent (A-) kinase consensus phosphorylation site also present in other *Shaker*-class channels. The conservation of this site in an otherwise divergent region of the protein suggests that the conservation is significant (*5*, *7-11*). In vertebrate K^+ channels this A-kinase site overlaps a consensus site for CaM II kinase phosphorylation. Consensus sequences for phosphorylation and N-glycosylation specific to each channel are indicated in Fig. 2.

It is apparent from an inspection of the sequences, both in the core regions and in the amino and carboxyl domains, that huKI and huKIV share more similarities with each other than either does with huKII. The differences are especially prominent in the N-terminal regions where the sequence of huKII shows essentially no similarity to either of the other channels. The N-terminal domain of the huKII protein is about 140 amino acid residues longer than huKI or huKIV (Fig 1; Fig 2b) and contains an unusual string of 15 acidic residues: one aspartic acid and 14 glutamic acid residues. The GenBank database contains 9 other sequences with 9 of 10 consecutive residues being glutamic acid. One of these sequences is the the *Electrophorus* sodium channel. In this protein, the polyglutamic acid sequence is present between homology domains II and III (*16*). The significance of this sequence is not known in either the eel channel or in huKII, but it is possible that the charged groups affect channel activity in some way. The carboxy- and amino-terminal regions of *Shaker* K^+ channels confer specific inactivation properties to the channels; it is conceivable that charged residues at the amino terminus of huKII may play a similar role (*6*).

Each of the human K^+ channel genes huKI to huKIV has a specific identified rat homolog (*7-11*) (Table 1). The alignments in Fig. 2 show remarkable similarities in sequence: RCK1 and huKI differ in only 10 amino acid residues; RCK4 and huKII differ in about 18 residues (primarily in the large amino-domain; see Fig. 2B legend); RBK2 and huKIV vary in only 3 amino acid residues. More sequences differences are observed between rat and human cytochrome C proteins, that are among the most highly conserved of metabolic enzymes. It is clear from these data, that this K^+ channel family diversified prior to the mammalian radiation. The high degree of conservation of K^+ channel sequences argues that each of these proteins has a specific function that is conserved among mammals. The conservation of the "variable" N- and C- termini further implies that these sequences also have specific, conserved, functions. The striking sequence homologies also

suggest that human K^+ channel genes and their rat homologs may be expressed in homologous cells. It is particularly interesting that RCK4 is expressed not only in rat brain, but also in rat heart muscle (*10,11*). The corresponding human gene huKII was isolated from a brain cDNA library. It is possible that it is also expressed in human heart.

Functional properties of human channels:

HuKI, huKII and huKIV cDNAs encode functional voltage-gated potassium channels, whose properties were examined in the *Xenopus* oocyte expression system. The expression of functional huKII channels was somewhat surprising, as the huKII cDNA used in our experiments may not encode a full-length, wild-type protein; for these channels, translation probably begins at a downstream methionine residue, indicated in Fig. 2B, that might not be used *in vivo* [Fig. 2B]. mRNA, transcribed *in vitro* from human cDNA clones, was microinjected into *Xenopus* oocytes; the message was efficiently translated by the cell, and functional channels assembled in oocyte membrane were assayed by two-microelectrode voltage clamp (*17*). mRNA from huKI, huKII and huKIV induced voltage-sensitive outward currents in oocytes, with reversal potentials that varied with extracellular K^+ concentration in a manner expected for K^+-selective channels. All the human channels currents activated relatively slowly. For example, the time to half maximum current at 0 mV was 5, 14, and 27ms for huKI, huKII and huKIV respectively (Fig. 3). None of the expressed currents inactivated more than 10% over 500 ms at 0 mV. In these characteristics, the human channels resemble the delayed-rectifier subtype of potassium channels.

While analyzing the physiological properties of the human channels, several interesting sequence-activity hypotheses in potassium channels could be directly examined. Two regions in the potassium channel sequence, S4 and the "leucine-zipper" that is adjacent to S4 (Fig. 1), have been implicated in voltage-dependent gating. The most accepted models suggest that charged residues in S4 determine the slope of the

conductance-voltage curve; mutations in the leucine-zipper region have been shown to produce shifts in the voltage dependence of channel activation (*18,19*). Our results show that other sequences in the channels also play some role in voltage-dependent behaviours. The conductance-voltage (g-V) curves of the huK currents, normalized to the highest observed conductance in each case, are presented in Fig. 4, and the data summarized in Table 2. In a sequence of increasing depolarizations, currents are first observed at about -50, -40 and -25 mV for huKI, huKII and huKIV, respectively. These differences in activation voltage cannot be explained by variations in S4 sequences or leucine-zipper sequences, since there is strict conservation of these sequences between huKI and huKIV. The g-V curves for huKI and huKIV are steeper than for huKII; these differences in the slope of the g-V curves cannot be ascribed to differences in charge of S4 since huKII differs in only 2 uncharged residues in this region (*18*).

We examined the sensitivities of huK currents to charybdotoxin (CTX, a constituent of scorpion venom that blocks a subgroup of K^+channels) (Table 2; *17*). There is good biophysical evidence that positively charged CTX binds, with largely electrostatic forces, to sequences close to the mouth of potassium channels (*20*). The region between H4 and H5 of *Drosophila Shaker* channels has been shown to interact with CTX and has therefore been postulated to be close to the channel pore (*20, 21*). Analysis of huKI and huKIV suggests that other extracellular loops must also be involved in the interaction with CTX and thus also contribute to the mouth of the channel. HuKIV has a higher affinity than huKI for CTX although the H4-H5 region is more negatively charged in huKI. While 10nM CTX reduces huKIV currents about 50%, huKI is essentially insensitive. Analysis of chimeric channels containing sequences from both huKI and huKIV should allow demarcation of other regions of the protein involved in CTX binding. It is a possibility, however, that some post-translational modification, specific to huKI, occludes a high-affinity CTX binding site in huKI channels.

We also examined the effects of other pharmacological agents on the human channels (Table 2; *17*). TEA (tetraethyl ammonium) and 4AP (4-aminopyridine) are well known K^+ channel blockers. The ED_{50} values (concentration for 50% inhibition of current) for 4AP are about 1mM for all the channels. The ED_{50} values of TEA are considerably more variable: huKI is sensitive (20 mM); huKII and huKIV are relatively insensitive (>50 mM). We were however surprised to find that DIDS (an isothiocyanate that covalently modifies reactive lysine residues) alters both the voltage dependence and the kinetics of all our human K^+ channels. This is specifically noteworthy because DIDS is often used as a Cl^- channel blocker to eliminate Cl^- conductances endogenous to oocyte membrane. An example of this effect is shown in Fig. 3C and 3D where the currents observed with huKIV with and without DIDS is shown. These effects are too large to be accounted for simply by the blocking of the small endogenous Cl^- currents. It is not clear, however, that the effects we observe are due to a direct effect on the channel molecules. There have been previous reports of isothiocyanates affecting the properties of potassium channel in neuronal membrane [*22*].

Interspecific comparisons of human-channel properties:

The physiological properties of human channels are largely comparable to those reported for their rodent homologs. HuKI and RCK1 are similar to each other in their voltage-dependent properties as might be expected from their similar sequences. HukIV activates more slowly than RCK5, and its voltage dependence is shifted to about 20mV more positive than RCK5. The observed differences between huKIV and RCK5 may easily be ascribed to sequence variations, some of which are in otherwise highly conserved regions in H3 and S4 [Fig 2B]. It has been previously shown that very subtle substitutions in conserved regions of ion-channels can cause dramatic differences in physiology [19]. Biological interpretations of the different voltage-dependences of huKIV and RCK5 are probably premature, especially as the sequence of the RCK5 channel is different in two

independent reports. Some of these observed sequence variations may easily be cloning artefacts generated during *in vitro* manipulations.

The most dramatic difference between the properties of a human channel and those of its rodent homolog is between huKII and RCK4. However, since our huKII product is probably missing about 100 aminoacids at the N- terminus, that are present in RCK4, a real comparison cannot be made. In fact, the differences between huKII and RCK4 are analogous to deletions from the N- terminus of *Drosophila Shaker* channels which drastically reduce the rate of inactivation but have no effect on voltage dependence or K^+ selectivity [*23*]. HuKII and RCK4, are similar in their voltage dependence of activation; however, while the rat channel inactivates with a halftime of about 20 ms, currents from our truncated huKII do not inactivate over several hundred milliseconds (Fig. 3). It is interesting that translation beginning at an alternative methionine codon proceeds efficiently and produces a distinctive functional channel in oocytes; however, the *in vivo* significance of this channel, if any, is not clear.

It should be noted that all the electrophysiological data reported here, were obtained by two-microelectrode voltage clamp of *Xenopus* oocytes. These are compared to data on rodent channels obtained by clamping large cell-attached patches of oocytes. While it is conceivable that the physiological differences observed in the huKI/RCK1 and huKIV/RCK5 pairs reflect technical limitations of one or both systems, it is not obvious to us that this is so.

The major pharmacological differences that we observe are that all human channels are less sensitive to TEA and CTX than their rat homologs. For example, huKI appears to be at least 20 fold less sensitive to TEA than RCK1; it is at least 5 fold less sensitive to CTX. These differences may reflect the fact that extracellular loops most likely to be involved in ligand binding are among the few divergent regions of the proteins. However, it remains possible that reagents from alternative sources do not have identical potencies; and that small variations in procedures followed in different laboratories, could lead to the

observed differences in pharmcological sensitivities. In either case, the observations are intriguing enough to warrant a careful comparison of rat and human channels under identical conditions, in the same laboratory. If the pharmacological differences are indeed *bona fide* variations, it may indicate that, in general, high affinity ligands developed specifically to a rat channel may not share the same affinity for its human homolog; this generalization has implications for clinical drug design.

The huKII gene is very similar in sequence to a recently identified rat K^+ channel gene called RCK4 which is expressed in brain and heart (10,11). RCK4 currents in *Xenopus* oocytes are similar to a transient outward current present in dog ventricular myocytes, rabbit AV node cells and sheep Purkinje fibers. The transient current in these cardiac cells is involved in determining the plateau voltage that follows a fast spike in the cardiac action potential; in this way it indirectly affects the activation of other currents that activate at the plateau potential. If the full-length huKII protein forms an inactivating K^+ channel, it may play a similar role in human heart. This would be especially significant as modifiers of K^+ channel activity are used to control cardiac arrhythmias (4,24). The isolation of a cardiac K^+ channel gene would facilitate the development of specific high affinity ligands to the channel that may be of clinical use (11,25).

Mutant *Shaker* alleles in *Drosophila* may remove an entire gene encoding a family of potassium channels. Such mutations cause neurological and neuromuscular defects in the animal without causing serious loss of viability. It is possible that defects in K^+ channel genes could similarly lead to non-lethal heritable diseases in humans. Some of these could be associated with particular myopathies or neural disorders. A careful cytogenetic mapping of these human K^+ channel genes is in progress. Preliminary evidence suggests that the genes all map to the group of chromosomes 9-12 that are not distinguished during single-laser chromosome sorting [*26*] (V. Sheffield, R. Lebo, et al., unpublished results). This may indicate that the genes are closely linked on a particular chromosome as appears to be the case with human sodium channel genes [*27*].

References.

1. B. Hille, *Ionic channels of excitable membrane* (Sinauer, Sunderland MA, 1984). B. Rudy, *Neurosci.* 25, 729-749 (1988). W. A. Catterall, *Science* 242, 50-60 (1988).

2. I. S. Cohen, N. B. Datyner, G. A. Gintant, R. P. Kline, in *The heart and the cardiovascular system; scientific foundations*. H. A. Fozzard, E. Haber, R. B. Jennings, A. M. Katz, H. E. Morgan, eds. (Raven Press, New York, 1986), pp. 637-670.

3. T. J. Carew, C. L. Sahley, *Ann. Rev. Neurosci.* 9, 435-487 (1986). L. K. Kaczmarek, in *Advances in Second Messenger and Phosphoprotein Research* P. Greengard, G. A. Robison, eds. (Raven Press, New York, 1988), pp. 113-138.

4. N. B. Standen *et al.*, *Science* 245, 177-180 (1989). A. L. Wit, M. R. Rosen, in *The heart and cardiovascular system: scientific foundations*. H. A. Fozzard, E. Haber, R. B. Jennings, A. M. Katz, H. E. Morgan, eds. (Raven Press, New York, 1986), pp. 1449-1490.

5. A. Kamb, L. E. Iverson, M. A. Tanouye, *Cell* 50, 405-413 (1987). D. M. Papazian, T. L. Schwarz, B. L. Tempel, Y. N. Jan, L. Y. Jan, *Science* 237, 749-753 (1987). B. L. Tempel, D. M. Papazian, T. L. Schwarz, Y. N. Jan, L. Y. Jan, *Science* 236, 770-775 (1987). A. Baumann *et al.*, *EMBO J.* 6, 3419-3429 (1987). A. Kamb, J. Tseng-Crank, M. A. Tanouye, *Neuron* 1, 421-430 (1988). O. Pongs *et al.*, *EMBO J.* 7, 1087-1096 (1988). T. L. Schwarz, B. L. Tempel, D. M. Papazian, Y. N. Jan, L. Y. Jan, *Nature* 331, 137-142 (1988).

6. L. E. Iverson, M. A. Tanouye, H. A. Lester, N. Davidson, B. Rudy, *Proc. Natl. Acad. S ci .U.S.A* 85, 5723-5727 (1988). L. C. Timpe, Y. N. Jan, L. Y. Jan, *Neuron* 1, 659-667 (1988). L. E. Iverson, B. Rudy, (Submitted for publication.). L. C. Timpe *et al.*, *Nature* 331, 143-145 (1988).

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

8. A. Baumann, A. Grupe, A. Ackerman, O. Pongs, *EMBO J.* 7, 2457-2463 (1988). M. J. Christie, J. P. Adelman, J. Douglass, A. J. North, *Science* 244, 221-224 (1989).

9. D. McKinnon, *JBC* 264, 8230-8236 (1989).

10. W. Stuhmer *et al.*, *EMBO J.* 8, 3235-3244 (1989).

11. J. Tseng-Crank, G.-N. Tseng, M. A. Tanouye, (Submitted for publication.).

12. G. C. Frech, A. M. J. VanDongen, G. Schuster, A. M. Brown, R. H. Joho, *Nature* 340, (1989).

13. A. Kamb, M. Weir, B. Rudy, H. Varmus, C. Kenyon, *Proc. Natl. Acad. Sci. U.S.A.* 86, 4372-4376 (1989).

14. Three cDNA libraries screened were gifts from Dr. Celia Campagnoni and Dr. Carmie Puckett. They were constructed in lambda gt11 phage from poly A+ mRNA

isolated from human fetal spinal cord, human brain stem and human basal ganglia respectively. The brain stem and basal ganglia library were prepared from autopsy tissue taken from a one day old patient (C. Puckett, J. Kamholz and R. A. Lazzarini). About 2 x 10^6 recombinant clones were screened from each library. A total of 5 cDNA clones were isolated for huKI, 4 for huKII, 3 for huKIII, 2 for huKIV and 7 for huKV. The human genomic library screened was constructed by Dr J. Weis.

15. K. G. Chandy, C. Williams, R. H. Spencer, B. Tempel, G. A. Gutman, *Science*. In Press (1990).

16. M. Noda *et al.*, *Nature* 312, 121-127 (1984).

17. K^+ channel cDNAs were subcloned into pGEMA, a vector provided by Dr. Rick Swanson of the Merck Sharpe Dohme Research Labs. pGEMA is a modified form of pGEM9zf- (Promega) with stretch of 40 A-T base pairs inserted just upstream of the NotI and Tth111I sites; thus, *in vitro* transcription of plasmid DNA linearized with NotI (or Tth111I) using T7 RNA polymerase generates polyadenylated cRNA. Polyadenylation appeared to increase both translatability and stability of the RNA. Capped, polyadenylated RNA (1-5 ng) was microinjected into stageV or stage VI Xenopus laevis oocytes. Ion currents were recorded using a standard two-electrode voltage clamp. The electrodes were filled with 3M KCl and had tip resistances of 0.7 to 1.2 MΩ. All experiments were carried out at room temperature (20-22C). Current signals were low-pass filtered at 3KHz. Voltage and current signals were digitized and analyzed using the pCLAMP package (Axon Instruments, Burlingame, CA). Leakage current was estimated from the average response to 10 mV steps from -90 mV in a voltage range where time-dependant currents were not activated. No K^+-selective currents were observed in uninjected oocytes. Data analysis was carried out using the pCLAMP package as described in L. E. Iverson, M. A. Tanouye,

H. A. Lester, N. Davidson, B. Rudy, *Proc. Natl. Acad. Sci. U.S.A.* 85, 5723-5727 (1988). Pharmacological sensitivities to 4AP and TEA were assayed at a test potential of 0mV after perfusing with ND96 solution containing specified concentrations of ligand for 10'. Charybdotoxin samples were obtained from two independent sources; one was a generous gift from Ana Maria Garcia of the Merck Sharpe Dohme Research Labs at Rahway, New Jersey; the other was purchased commercially from Alamone Labs, Jerusalem, Israel. Solutions of CTX were made up in 0.1%BSA., 100mM NaCl, 10mMTris (7.5), 1mM EDTA, at a concentration of 10μM, added directly to the bath and recordings made 10' after addition. The activities of the two CTX samples were indistinguishable in our assays.

18. W. Stuhmer *et al.*, *Nature* 339, 597-603 (1989). D. M. Papazian, L. C. Timpe, Y. N. Jan, L. Y. Jan, *Soc. Neurosci. Abs.* 15, 337 (1989).

19. K. J. McCormack *et al.*, *Nature* 340, (1989). K. J. McCormack et al., Soc. Neurosci. Abs. 15, 337 (1989). V. J. Auld *et al.*, *Proc. Natl. Acad. Sci. U.S.A.*, in press.

20. R. Mackinnon, C. Miller, *Biochemistry* 28, 8087-8092 (1989).

21. R. MacKinnon, C. Miller, *Science* 245, 1382-1385 (1989).

22 D.C. Eaton and M.S. Brodwick, *Biophysical Journal* 25, 305a (1979). I. Inouye, *J. Gen. Physiol.* 88, 507-520 (1986). G.S. Oxford and P.K. Wagoner, *J. Physiol.* 410, 587-612 (1989)

23. T. Hoshi, W. N. Zagotta, R. W. Aldrich, *Soc. Neurosci. Abs.* 15, 338 (1989).

24. E. Carmiliet, *J. Pharmacol. Exp. Ther.* 232, 817-825 (1985).

25. H. A. Lester, *Science* 241, 1057-1063 (1988).

26. R. V. Lebo, B. D. Bruce, *Methods Enzymol.* 151, 292-313 (1987).

27. M. Litt *et al., Genomics* 5, 204-208 (1989).

28. T. Takumi, H. Ohkubo, S. Nakanishi, *Science* 242, 1042-1045 (1988). T. Murai, A. Kakizuka, T. Takumi, H. Ohkubo, S. Nakanishi, *Biophys. Biochem. Res. Commun.*161, 176-181 (1989).

29. Abel, T., Maniatis T., *Nature* 341, 24-25 (1989).

30. S. G. Miller, B. L. Patton, M. B. Kennedy, *Neuron* 1, 593-604 (1988). C. S. Hubbard, R. J. Ivatt, *Ann. Rev. Biochem.* 50, 555-583 (1981). E. G. Krebs, J.-A. Beavo, *Ann. Rev. Biochem.* 48, 923-959 (1979).

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

32. We thank Carmie Puckett for DNA libraries; R. Lebo, Val Sheffield and J. Tseng-Crank for communicating their results to us prior to publication; K. J. McCormack and L. Iverson for stimulating discussions; R. McMahon for excellent technical assistance; and members of H. Lester's laboratory for generous advice and assistance with electrophysiological methods. This work was supported by USPHS grants NS21327 and

GM42824 to M.A.T., a Markey Charitable Trust Graduate Fellowship to M.R. and the USPHS grant GM29836 to H. Lester.

Figure 1: An alignment of deduced amino acid sequences from huKI, huKII and huKIV cDNAs.

Dashes indicate aminoacid identity; gaps have been inserted to maximize sequence similarities. The start codons in huKI and huKIV are the most upstream, in-frame, methionine codons found in our cDNA clones. Stop codons exist 5' to this ATG sequence in all three reading frames. The putative start and the first 50 amino acid residues of sequence in huKII were inferred from genomic DNA. These features are consistent with the highly homologous RCK4 (RHK1) sequence (Fig. 2B).

Bars indicate seven potentially membrane-spanning segments H1-H6 and S4 . It is worth noting that H5 and H6 are separated by only three amino acid residues; in addition, H5 contains residues predicted to be incompatible with a hydrophobic, alpha-helical structure. These observations indicate that H5 might not span the membrane. The S4 segments in these proteins are composed of seven iterations of the unit X-Y-Arg/Lys, where X and Y are usually hydrophobic amino acids. Such segments are found in all voltage-gated ion channels cloned to date with one possible exception (*28*). S4 segments are thought to be membrane-spanning units that sense and respond to changes in transmembrane potential (*16*). Their involvement in voltage sensing has been demonstrated in sodium and K^+ channels (*18*).

Inverted triangles indicate leucines in a heptad repeat that occurs between S4 and H4. Such sequences (leucine zippers) have been shown to be involved in subunit association in several DNA binding proteins (*29*). They might serve similar functions in channels (*19*). The shaded region indicates the approximate boundaries of the core region. Circles indicate putative phosphorylation sites conserved among these proteins. Arrows point to conserved N-linked glycosylation sites between H1 and H2, and prior to H1. The latter is probably intracellular and is therefore unlikely to be significant. Consensus sequences for phosphorylation and N-linked glycosylation are as described in (*30*)

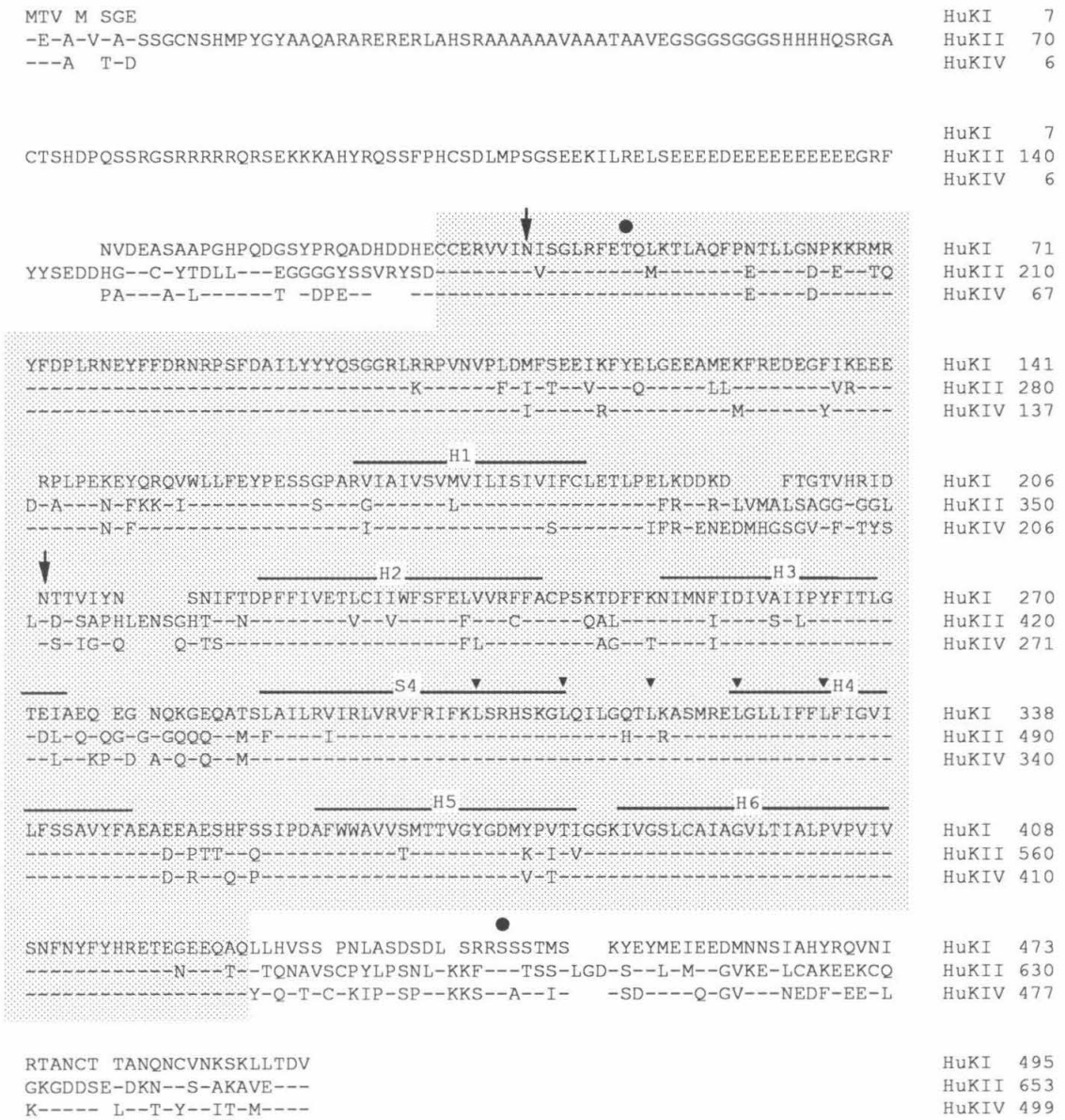

MTV M SGE HuKI 7
-E-A-V-A-SSGCNSHMPYGYAAQARARERERLAHSRAAAAAAVAAATAAVEGSGGSGGGSHHHHQSRGA HuKII 70
---A T-D HuKIV 6
HuKI 7
CTSHDPQSSRGSRRRRRQRSEKKKAHYRQSSFPHCSDLMPSGSEEKILRELSEEEEDEEEEEEEEEGRF HuKII 140
HuKIV 6
NVDEASAAPGHPQDGSYPRQADHDDHECCERVVINISGLRFETQLKTLAQFPNTLLGNPKKRMR HuKI 71
YYSEDDHG--C-YTDLL---EGGGGYSSVRYSD--------V--------M-------E----D-E--TQ HuKII 210
PA---A-L------T -DPE-- ----------E----D------ HuKIV 67
YFDPLRNEYFFDRNRPSFDAILYYYQSGGRLRRPVNVPLDMFSEEIKFYELGEEAMEKFREDEGFIKEEE HuKI 141
----------K------F-I-T--V---Q-----LL--------VR--- HuKII 280
----------I-----R----------M------Y----- HuKIV 137
H1
RPLPEKEYQRQVWLLFEYPESSGPARVIAIVSVMVILISIVIFCLETLPELKDDKD FTGTVHRID HuKI 206
D-A---N-FKK-I----------S---G------L----------FR--R-LVMALSAGG-GGL HuKII 350
------N-F----------I----------S-------IFR-ENEDMHGSGV-F-TYS HuKIV 206
H2 H3
NTTVIYN SNIFTDPFFIVETLCIIWFSFELVVRFFACPSKTDFFKNIMNFIDIVAIIPYFITLG HuKI 270
L-D-SAPHLENSGHT--N--------V--V-----F---C-----QAL-------I----S-L------- HuKII 420
-S-IG-Q Q-TS----------FL---------AG--T----I---------- HuKIV 271
S4 H4
TEIAEQ EG NQKGEQATSLAILRVIRLVRVFRIFKLSRHSKGLQILGQTLKASMRELGLLIFFLFIGVI HuKI 338
-DL-Q-QG-G-GQQQ--M-F----I----------H--R---------- HuKII 490
--L--KP-D A-Q-Q--M---------- HuKIV 340
H5 H6
LFSSAVYFAEAEEAESHFSSIPDAFWWAVVSMTTVGYGDMYPVTIGGKIVGSLCAIAGVLTIALPVPVIV HuKI 408
----------D-PTT--Q----------T---------K-I-V---------- HuKII 560
----------D-R--Q-P----------V-T---------- HuKIV 410
SNFNYFYHRETEGEEQAQLLHVSS PNLASDSDL SRRSSSTMS KYEYMEIEEDMNNSIAHYRQVNI HuKI 473
----------N---T--TQNAVSCPYLPSNL-KKF---TSS-LGD-S--L-M--GVKE-LCAKEEKCQ HuKII 630
----------Y-Q-T-C-KIP-SP--KKS--A--I- -SD----Q-GV---NEDF-EE-L HuKIV 477
RTANCT TANQNCVNKSKLLTDV HuKI 495
GKGDDSE-DKN--S-AKAVE--- HuKII 653
K----- L--T-Y--IT-M---- HuKIV 499

Figure 2: Alignments between human and rat K^+ channel homologs.

Dashes indicate aminoacid identity; gaps have been inserted to maximize sequence similarities. Arrows indicate putative N-linked glycosylation sites. Circles indicate consensus sequences for phosphorylation by protein kinases. Bars indicate potentially transmembrane segments.

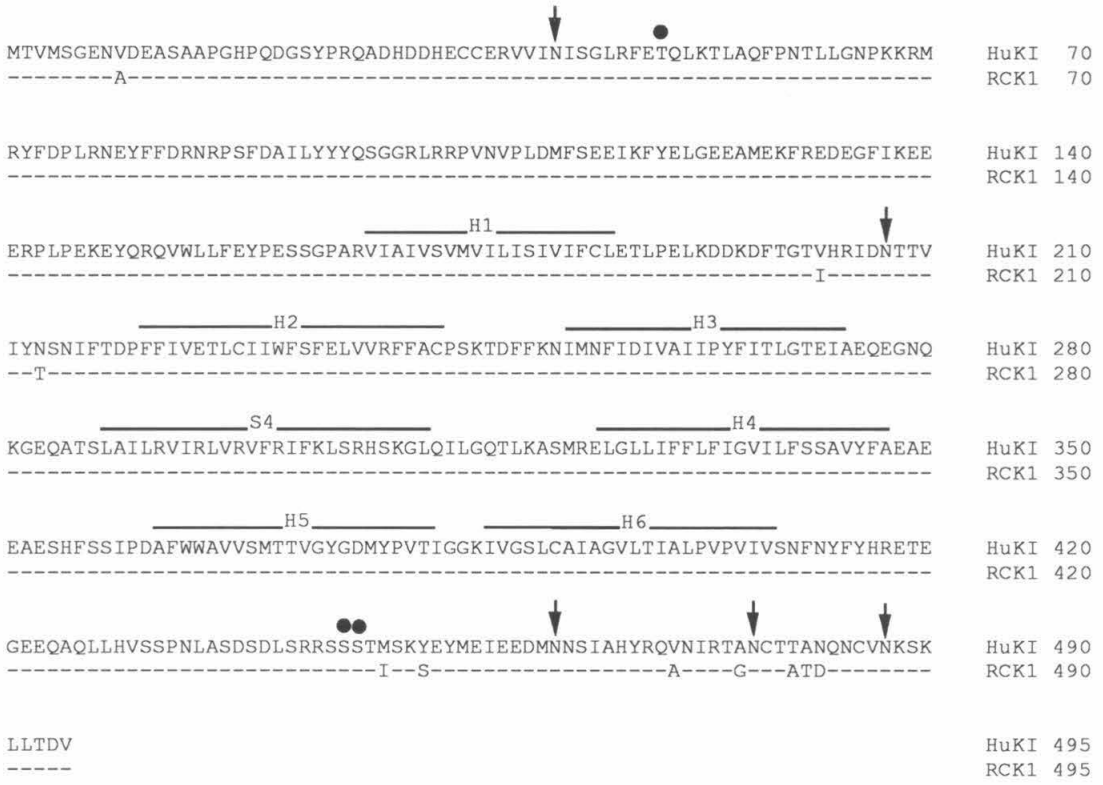
A) HuKI and RCK1 (RBK1) alignment.

B) HuKII and RCK4 (RHK1) alignment. The methionine residue indicated by a * corresponds to the probable start used in the *Xenopus* oocyte translation of microinjected huKII mRNA; as our huKII cDNA did not contain sequences encoding the first 50 N-terminal residues, this may not be a start codon used *in vivo* .

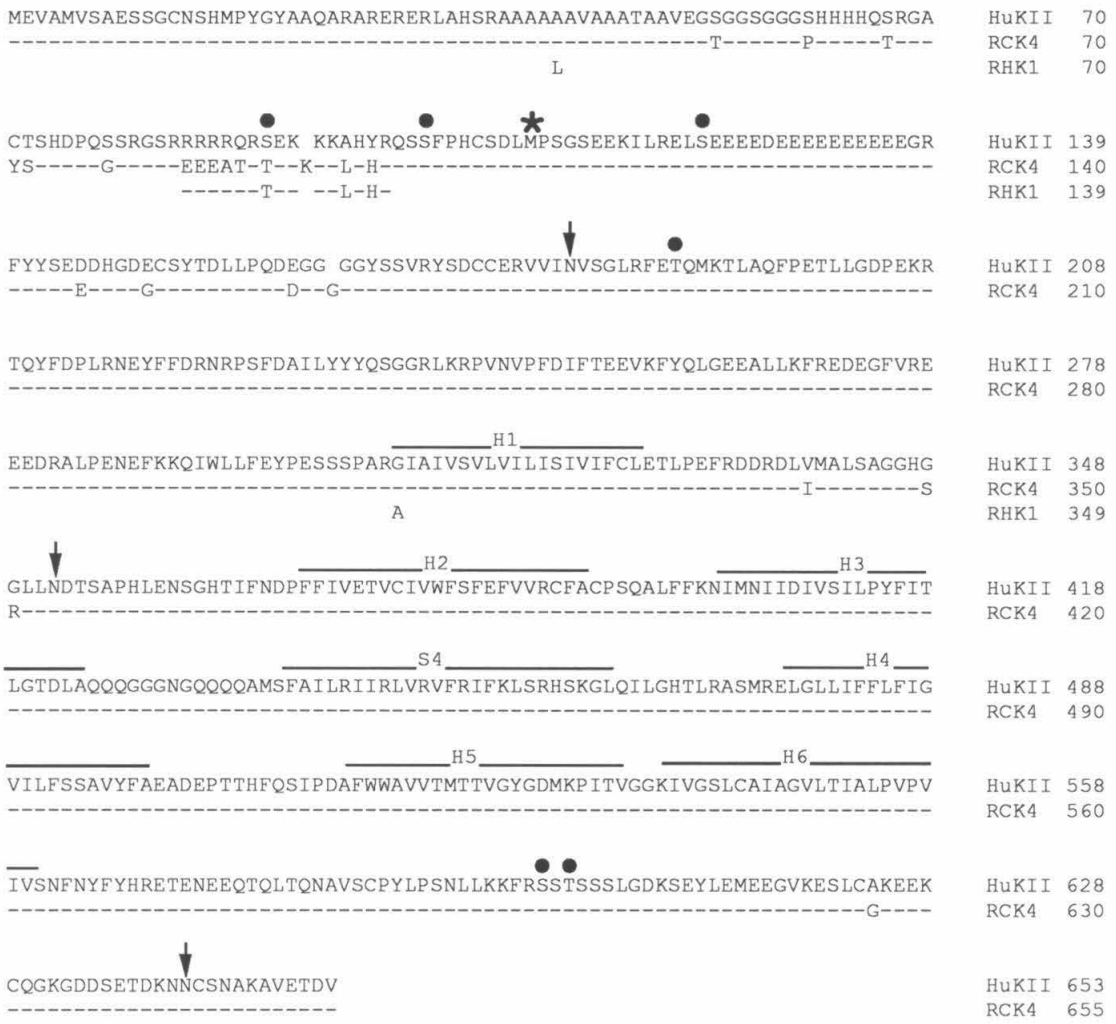
While RCK4 and RHK1 most likely represent the same gene their reported sequences are different at the indicated positions. The first consensus sequence for phosphorylation indicated is present in the reported RHK1 sequence but not in RCK4. This is due to two frame shifts present in the RCK4 nucleotide sequence compared to RHK1. The number of sequence differences, between huKII and RCK4, mentioned in the text, assumes that the frameshifts are sequence errors in RCK4.

C) HuKIV and RBK2 (RCK5) alignment. RCK5 and RBK2 are likely to be the same gene; however their reported sequences differ at the indicated residues.

A


MTVMSGENVDEASAAPGHPQDGSYPRQADHDDHECCERVVINISGLRFETQLKTLAQFPNTLLGNPKKRM HuKI 70
--------A---------- RCK1 70
RYFDPLRNEYFFDRNRPSFDAILYYYQSGGRLRRPVNVPLDMFSEEIKFYELGEEAMEKFREDEGFIKEE HuKI 140
---------- RCK1 140
H1
ERPLPEKEYQRQVWLLFEYPESSGPARVIAIVSVMVILISIVIFCLETLPELKDDKDFTGTVHRIDNTTV HuKI 210
----------I-------- RCK1 210
H2 H3
IYNSNIFTDPFFIVETLCIIWFSFELVVRFFACPSKTDFFKNIMNFIDIVAIIPYFITLGTEIAEQEGNQ HuKI 280
--T---------- RCK1 280
S4 H4
KGEQATSLAILRVIRLVRVFRIFKLSRHSKGLQILGQTLKASMRELGLLIFFLFIGVILFSSAVYFAEAE HuKI 350
---------- RCK1 350
H5 H6
EAESHFSSIPDAFWWAVVSMTTVGYGDMYPVTIGGKIVGSLCAIAGVLTIALPVPVIVSNFNYFYHRETE HuKI 420
---------- RCK1 420
GEEQAQLLHVSSPNLASDSDLSRRSSSTMSKYEYMEIEEDMNNSIAHYRQVNIRTANCTTANQNCVNKSK HuKI 490
----------I--S----------A----G---ATD-------- RCK1 490
LLTDV HuKI 495
----- RCK1 495

B


MEVAMVSAESSGCNSHMPYGYAAQARARERERLAHSRAAAAAAVAAATAAVEGSGGSGGGSHHHHQSRGA HuKII 70
T P T RCK4 70
L RHK1 70
CTSHDPQSSRGSRRRRRQRSEK KKAHYRQSSFPHCSDLMPSGSEEKILRELSEEEEDEEEEEEEEEGR HuKII 139
YS G EEEAT T K L H RCK4 140
T L H RHK1 139
FYYSEDDHGDECSYTDLLPQDEGG GGYSSVRYSDCCERVVINVSGLRFETQMKTLAQFPETLLGDPEKR HuKII 208
E G D G RCK4 210
TQYFDPLRNEYFFDRNRPSFDAILYYYQSGGRLKRPVNVPFDIFTEEVKFYQLGEEALLKFREDEGFVRE HuKII 278
RCK4 280
H1
EEDRALPENEFKKQIWLLFEYPESSSPARGIAIVSVLVILISIVIFCLETLPEFRDDRDLVMALSAGGHG HuKII 348
I S RCK4 350
A RHK1 349
H2 H3
GLLNDTSAPHLENSGHTIFNDPFFIVETVCIVWFSFEFVVRCFACPSQALFFKNIMNIIDIVSILPYFIT HuKII 418
R RCK4 420
S4 H4
LGTDLAQQQGGNGQQQQAMSFAILRIIRLVRVFRIFKLSRHSKGLQILGHTLRASMRELGLLIFFLFIG HuKII 488
RCK4 490
H5 H6
VILFSSAVYFAEADEPTTHFQSIPDAFWWAVVTMTTVGYGDMKPITVGGKIVGSLCAIAGVLTIALPVPV HuKII 558
RCK4 560
IVSNFNYFYHRETENEEQTQLTQNAVSCPYLPSNLLKKFRSSTSSSLGDKSEYLEMEEGVKESLCAKEEK HuKII 628
G RCK4 630
CQGKGDDSETDKNNCSNAKAVETDV HuKII 653
RCK4 655

C

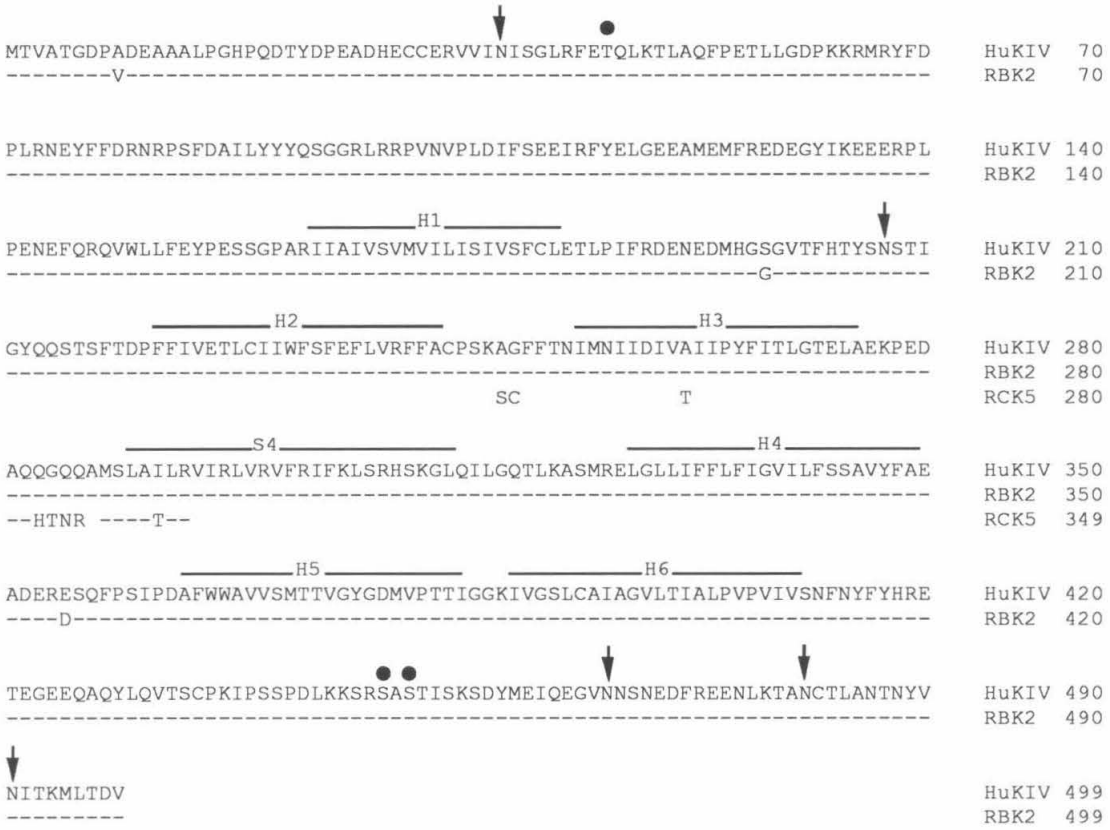

MTVATGDPADEAAALPGHPQDTYDPEADHECCERVVINISGLRFETQLKTLAQFPETLLGDPKKRMRYFD HuKIV 70
--------V---------- RBK2 70
PLRNEYFFDRNRPSFDAILYYYQSGGRLRRPVNVPLDIFSEEIRFYELGEEAMEMFREDEGYIKEEERPL HuKIV 140
---------- RBK2 140
H1
PENEFQRQVWLLFEYPESSGPARIIAIVSVMVILISIVSFCLETLPIFRDENEDMHGSGVTFHTYSNSTI HuKIV 210
----------G---------- RBK2 210
H2 H3
GYQQSTSFTDPFFIVETLCIIWFSFEFLVRFFACPSKAGFFTNIMNIIDIVAIIPYFITLGTELAEKPED HuKIV 280
---------- RBK2 280
SC T RCK5 280
S4 H4
AQQGQQAMSLAILRVIRLVRVFRIFKLSRHSKGLQILGQTLKASMRELGLLIFFLFIGVILFSSAVYFAE HuKIV 350
---------- RBK2 350
--HTNR ----T-- RCK5 349
H5 H6
ADERESQFPSIPDAFWWAVVSMTTVGYGDMVPTTIGGKIVGSLCAIAGVLTIALPVPVIVSNFNYFYHRE HuKIV 420
----D---------- RBK2 420
TEGEEQAQYLQVTSCPKIPSSPDLKKSRSASTISKSDYMEIQEGVNNSNEDFREENLKTANCTLANTNYV HuKIV 490
---------- RBK2 490
NITKMLTDV HuKIV 499
--------- RBK2 499

Figure 3: Expression of outward currents in *Xenopus* oocytes injected with *in vitro* transcribed huK RNAs.

Membrane potential was held at -90 mV, followed by a one second hyperpolarizing pulse to -120 mV, and then stepped to test potentials indicated beside each trace. Test pulses were applied at a frequency of one every 10 seconds. **A**) HuKI; **B**) huKII; **C**) huKIV. **D**) huKIV + DIDS. All of the channels were sensitive to DIDS, but huKIV was the most sensitive; the effect on huKIV was noticable at 10μM DIDS.

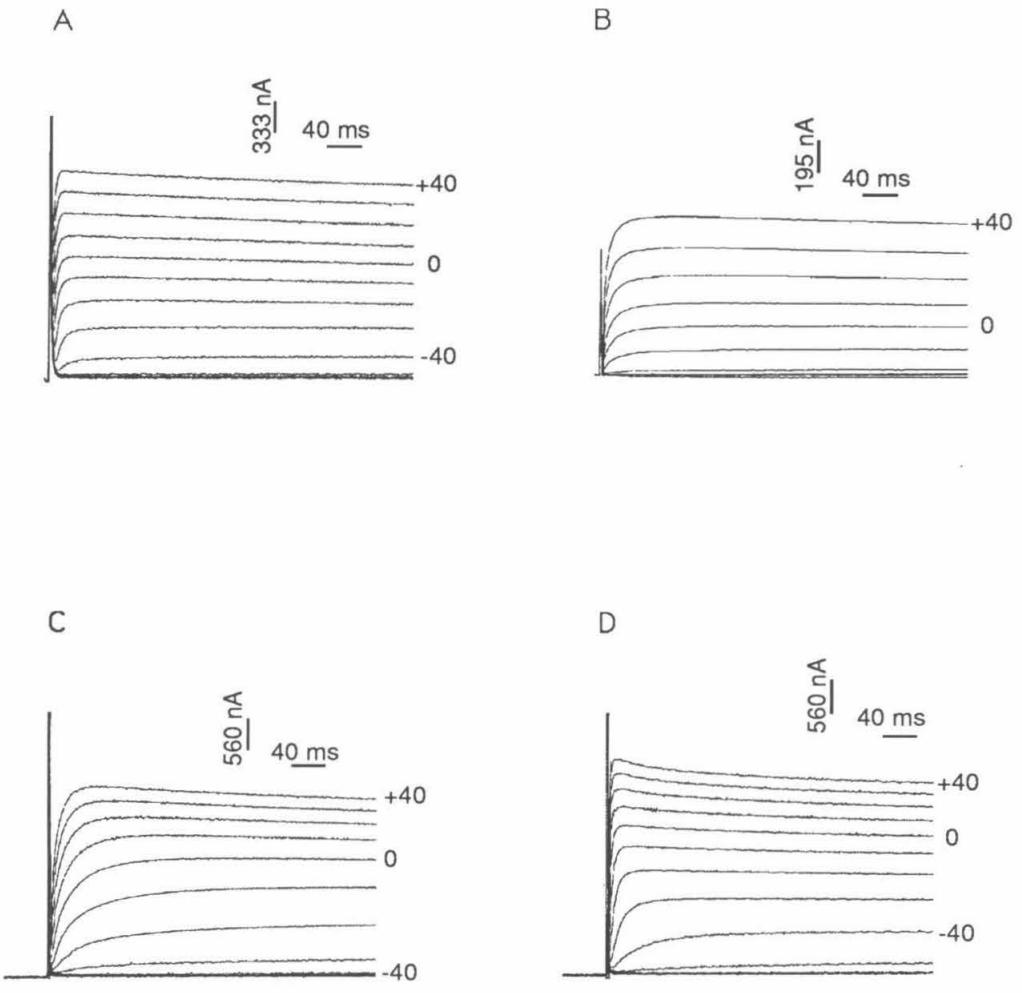
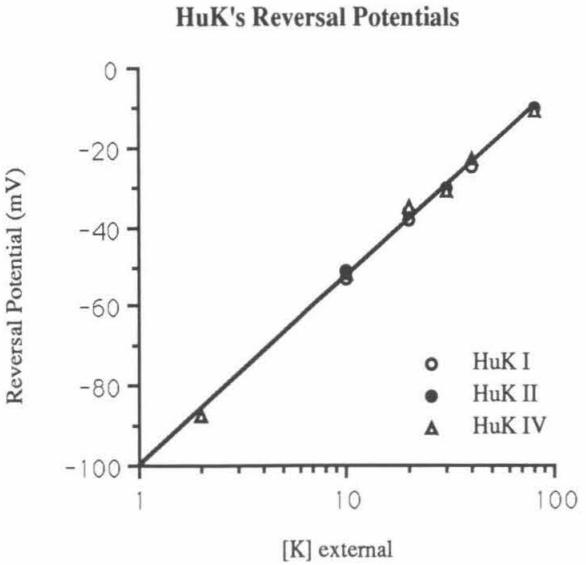

A
333 nA
40 ms
+40
0
-40
B
195 nA
40 ms
+40
0
C
560 nA
40 ms
+40
0
-40
D
560 nA
40 ms
+40
0
-40

Figure 4: K^+ conductance as a function of membrane potential.

A. Reversal potentials of human potassium channels as a function of extracellular potassium concentration. The plot is linear with a slope of approximately 50mV per 10-fold change in extracellular K+ concentration.

B. Conductances were derived from plateau currents and are expressed relative to the highest observed conductance for each oocyte. Driving force was calculated assuming a reversal potential of 80 mV. Values represent averages from at least five oocytes for each channel type.

A


HuK's Reversal Potentials
Reversal Potential (mV)
0
-20
-40
-60
-80
-100
1
10
100
[K] external
HuK I
HuK II
HuK IV

B

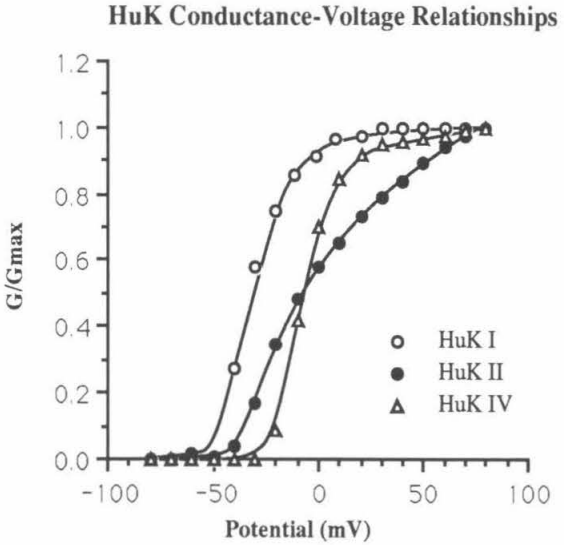

HuK Conductance-Voltage Relationships
G/Gmax
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-100
-50
0
50
100
Potential (mV)
HuK I
HuK II
HuK IV

Table 1: Members of a family of voltage-gated K^+ channels whose sequences have been reported to date.

All of these share an overall structural organization and significant sequence similarities. We have compiled sequence comparisons made by several groups including ours, and defined three distinct classes of K^+ channels within this family. Genes within a class show stronger sequence similarity to each other. In the best-conserved, core region, the sequence identity between two members in a given class is at least 70%. The identities shared between members of different classes is about 40%. K^+ channel genes in the same row represent homologs in the different species. These are essentially identical (>99% identity) within the "core" region. Most of the discussion in this paper deals with Class I or *Shaker*-class K^+ channels. HuKIII appears to be the human homolog of RCK3; however, the sequence is not complete. The slow delayed rectifier channel cloned by the Nakanishi group is not related in sequence to those listed above and may define a different family of K^+ channels (*28*).

Table 1:

Fly	Mouse	Rat	Human	Ref.
Class I: *Shaker* Class Potassum Channels				
Shaker				(*5*)
	MBK1	RCK1=RBK1	huKI	(*7, 8, 13*)
		RBK2=RCK5	huKIV	(*9, 10, 13*)
		RCK4=RHK1	huKII	(*10, 11, 13*)
		RCK3	huKIII	(*10*)
Class II:				
Shab				(*31*)
		drk1		(*12*)
Class III:				
Shaw				(*31*)

Table 2: Properties of huK channels in Xenopus oocytes[a].

[a] Values expressed as Mean ± SD with number of experiments in parantheses. [b] Potential at which conductance begins to rise. [c] Potential at which conductance has reached 1/2 its maximal value. [d] Measured at a 0 mV test pulse. [e] Concentration at which channel conductance is reduced by 1/2, monitoring a 0 mV test pulse. [f] At these concentrations, TEA becomes the major cation in the bathing medium and higher concentrations were not attempted.

Table 2:

Channel	Voltage of Activation (mV)[b]	Midpoint of activation curve (mV)[c]	Rise Time (ms)[d]	ED_{50} for 4-AP (mM)[e]	ED_{50} for TEA (mM)	ED_{50} for CTX (nM)
HuK I	-48±5.4 (5)	-30±6.3 (5)	5 (5)	1.1	20	»100
HuK II	-40±7.3 (3)	-5±8.7 (3)	14 (3)	0.80	>50	»100
HuK IV	-20±3.5 (11)	-5±4.2 (11)	27 (11)	1.0	>50	10

Appendix 1

Properties of full-length huKII channels in *Xenopus* oocytes.

Summary:

The huKII cDNA (hkII.2) described in Chapter 3 probably lacks about 150 nucleotides of coding sequence. Potassium-selective currents expressed in *Xenopus* oocytes from this truncated cDNA, inactivate very slowly, with a time constant much greater than one second. Full length cDNA from the homologous rat gene RCK4 (RHK1) encodes a rapidly inactivating potassium channel.

We have recently made a plasmid construct containing the complete coding sequence of huKII; we have also examined the properties of full length hukII channels in *Xenopus* oocytes. These huKII channels are similar to RCK4 in that the macroscopic current in frog oocytes inactivates rapidly, with a time constant of about 40ms at 20mV. Two major conclusions may be reached from this experiment: 1) The overall physiological properties of human and rat potassium channels are conserved; and 2) aminoacid sequences required for huKII channel inactivation, are located in the amino region, that is missing in channels expressed from cDNA hkII.2. Relevant data from these physiological experiments are shown in Figures 1, 2A and 2B.

Figure 1: Voltage-clamped huKII currents in *Xenopus* oocytes.

The oocytes were held at a potential -90mV. Following a prepulse for 2s at -120mV, to remove any residual inactivation, the voltage was stepped up to test potentials from -80mV to +120mV, at 10mV intervals. Transient, outward currents recorded at each test potential are shown in the figure as a function of time. The currents are carried by voltage-gated, potassium-selective, huKII RNA-induced channels [data not shown].

Voltage-clamped transient huKII currents in *Xenopus* oocytes

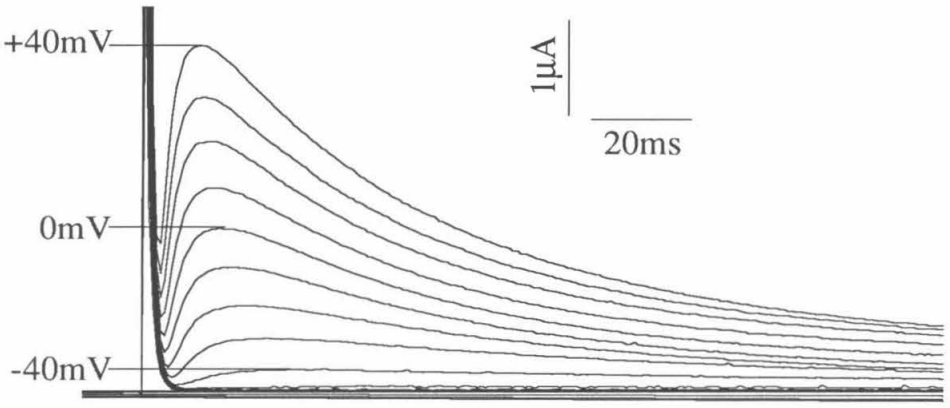
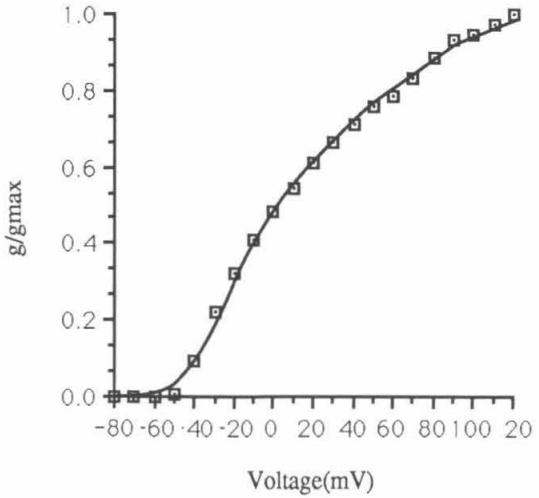


Figure 2: Voltage dependence of huKII currents.

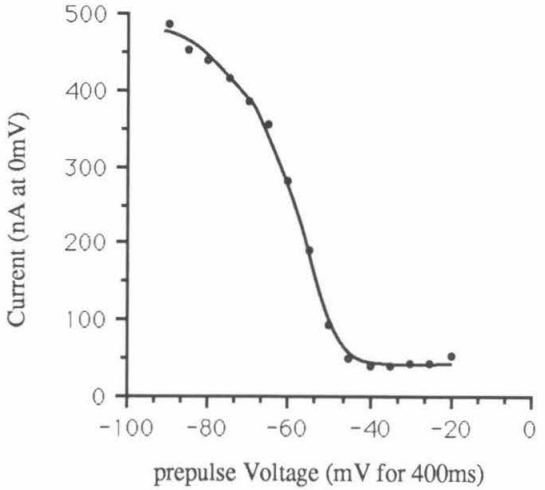
A. Voltage-dependence of channel activation: The membrane conductance to potassium, at each test potential in Fig. 1, was calculated (assuming a potassium equilibrium potential of -90mV). The ratio of this conductance (g) to the maximum observed conductance (gmax) at 120mV, is plotted as a function of voltage. The channel begins to activate at about -40mV; conductance does not appear to saturate, even at very positive (and unphysiological) potentials. These properties are not significantly different from those of truncated channels expressed from cDNA hkII.2 [Chapter 3].

B. Voltage dependence of steady-state inactivation: The oocyte expressing huKII currents was held at -90mV and then hyperpolarized to -120mV for 2s to remove residual inactivation. The membrane potential was then prepulsed at varying test potentials (from -90mV to -20mV, at 5mV intervals) for 400ms. The amplitude of currents recorded at 0mV, following the prepulse, is shown as a function of prepulse potential. Inactivation is essentially complete at -45mV; in this respect, and also in the relatively steep dependence of steady-stae inactivation on voltage, huKII channels resemble the "A-type" of potassium channels. The small currents observed, following prepulses at potentials more positive than -40mV, may either represent a non-inactivating component, or a small leakage current that has not been subtracted.

A. Conductance vs voltage



B. Steady-state inactivation



Appendix 2

Nucleotide sequences of cDNA from three human potassium-channel genes.

huKI

ATGACGGTGA TGTCTGGGGA GAACGTGGAC GAGGCTTCGG CCGCCCCGGG 50

CCACCCCCAG GATGGCAGCT ACCCCCGCCA GGCCGACCAC GACGACCACG 100

AGTGCTGCGA GCGCGTGGTG ATCAACATCT CCGGGCTGCG CTTCGAGACG 150

CAGCTCAAGA CCCTGGCGCA GTTCCCCAAC ACGCTGCTGG GCAACCCTAA 200

GAAACGCATG CGCTACTTCG ACCCCCTGAG GAACGAGTAC TTCTTCGACC 250

GCAACCGGCC CAGCTTCGAC GCCATCCTCT ACTACTACCA GTCCGGCGGC 300

CGCCTGCGGA GGCCGGTCAA CGTGCCCCTG GACATGTTCT CCGAGGAGAT 350

CAAGTTTTAC GAGTTGGGCG AGGAGGCCAT GGAGAAGTTC CGGGAGGACG 400

AGGGCTTCAT CAAGGAGGAG GAGCGCCCTC TGCCCGAGAA GGAGTACCAG 450

CGCCAGGTGT GGCTGCTCTT CGAGTACCCC GAGAGCTCGG GGCCCGCCAG 500

GGTCATCGCC ATCGTCTCCG TCATGGTCAT CCTCATCTCC ATCGTCATCT 550

TTTGCCTGGA GACGCTCCCC GAGCTGAAGG ATGACAAGGA CTTCACGGGC 600

ACCGTCCACC GCATCGACAA CACCACGGTC ATCTACAATT CCAACATCTT 650

CACAGACCCC TTCTTCATCG TGGAAACGCT GTGCATCATC TGGTTCTCCT 700

TCGAGCTGGT GGTGCGCTTC TTCGCCTGCC CCAGCAAGAC GGACTTCTTC 750

AAAAACATCA TGAACTTCAT AGACATTGTG GCCATCATTC CTTATTTCAT 800

CACCCTGGGC ACCGAGATAG CTGAGCAGGA AGGAAACCAG AAGGGCGAGC 850

AGGCCACCTC CCTGGCCATC CTCAGGGTCA TCCGCTTGGT AAGGGTTTTT 900

AGAATCTTCA AGCTCTCCCG CCACTCTAAG GGCCTCCAGA TCCTGGGCCA 950

GACCCTCAAA GCTAGTATGA GAGAGCTAGG GCTGCTCATC TTTTTCCTCT 1000

TCATCGGGGT CATCCTGTTT TCTAGTGCAG TGTACTTTGC CGAGGCGGAA 1050

GAAGCTGAGT CGCACTTCTC CAGTATCCCC GATGCTTTCT GGTGGGCGGT 1100

GGTGTCCATG ACCACTGTAG GATACGGTGA CATGTACCCT GTGACAATTG 1150

GAGGCAAGAT CGTGGGCTCC TTGTGTGCCA TCGCTGGTGT GCTAACAATT 1200

GCCCTGCCCG TACCTGTCAT TGTGTCCAAT TTCAACTATT TCTACCACCG 1250

AGAAACTGAG GGGGAAGAGC AGGCTCAGTT GCTCCACGTC AGTTCCCCTA 1300

ACTTAGCCTC TGACAGTGAC CTCAGTCGCC GCAGTTCCTC TACTATGAGC 1350

AAGTATGAGT ACATGGAGAT CGAAGAGGAT ATGAATAATA GCATAGCCCA 1400

TTATAGACAG GTCAATATCA GAACTGCCAA TTGCACCACA GCTAACCAAA 1450

ACTGCGTTAA TAAGAGCAAG CTACTGACCG ATGTTTAA 1488

huKII

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GCCTTATGGT TATGCTGCCC AGGCCCGGGC CCGGGAGCGG GAGAGGCTTG 100

CTCACTCCAG GGCAGCTGCA GCAGCTGCTG TTGCAGCGGC CACAGCTGCT 150

GTCGAAGGTA GCGGGGGTTC TGGTGGGGGC TCCCACCACC ACCACCAGTC 200

ACGCGGGGCC TGTACCTCCC ATGACCCTCA GAGCAGCCGG GGTAGTCGGA 250

GGAGGAGGCG ACAGCGGTCT GAGAAGAAGA AAGCCCACTA CCGGCAGAGC 300

AGCTTCCCTC ATTGCTCTGA CCTGATGCCC AGTGGCTCTG AGGAGAAGAT 350

CCTGAGGGAG CTGAGTGAGG AGGAGGAAGA TGAGGAGGAG GAGGAAGAGG 400

AGGAAGAGGA GGGAAGGTTT TACTATAGTG AAGATGACCA TGGTGATGAG 450

TGTTCCTACA CGGATCTGCT GCCTCAGGAT GAGGGCGGTG GCGGCTACAG 500

TTCAGTCCGC TACAGTGACT GTTGTGAACG TGTGGTGATA AATGTGTCAG 550

GCCTACGCTT TGAGACCCAA ATGAAAACTC TGGCCCAGTT TCCAGAGACT 600

TTGTTGGGAG ACCCTGAAAA GAGGACTCAG TACTTTGACC CTTTGCGCAA 650

TGAGTATTTT TTTGACAGGA ACCGCCCCAG CTTTGATGCC ATCTTGTATT 700

ATTATCAATC AGGAGGCCGC CTGAAGAGGC CAGTCAATGT CCCCTTTGAT 750

ATCTTCACTG AGGAGGTGAA GTTCTATCAG TTGGGGGAGG AGGCCCTGTT 800

GAAGTTTCGG GAGGACGAGG GCTTTGTGAG AGAAGAGGAA GACAGGGCCC 850

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GCGGATGAAC CTACTACCCA TTTCCAAAGC ATCCCAGATG CATTTTGGTG 1550

GGCTGTGGTG ACCATGACAA CTGTGGGCTA TGGGGACATG AAGCCCATCA 1600

CTGTAGGGGG CAAGATTGTC GGGTCCCTGT GTGCCATTGC GGGTGTCTTA 1650

ACCATTGCTT TGCCAGTGCC AGTGATTGTC TCTAACTTTA ACTATTTCTA 1700

CCACAGAGAG ACTGAAAATG AGGAACAGAC ACAGCTAACG CAGAATGCAG 1750

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AGTTAAGGAA TCTCTGTGTG CAAAGGAGGA GAAGTGTCAG GGAAAGGGGG 1900

ATGACAGTGA GACAGATAAA AACAACTGTT CTAATGCAAA GGCTGTGGAG 1950

ACTGATGTGT GA 1962

huKIV

ATGACAGTGG CCACCGGAGA CCCAGCAGAC GAGGCTGCTG CCCTCCCTGG 50

GCACCCACAG GACACCTATG ACCCAGAGGC AGACCACGAG TGCTGTGAGA 100

GGGTGGTGAT CAACATCTCA GGGCTGCGGT TTGAGACCCA GCTAAAGACC 150

TTAGCCCAGT TTCCAGAGAC CCTCTTAGGG GACCCAAAGA AACGAATGAG 200

GTACTTTGAC CCCCTCCGAA ATGAGTACTT TTTCGATCGG AACCGCCCTA 250

GCTTTGATGC CATTTTGTAC TACTACCAGT CAGGGGGCCG ATTGAGGCGA 300

CCTGTGAATG TGCCCTTAGA TATATTCTCT GAAGAAATTC GGTTTTATGA 350

GCTGGGAGAA GAAGCGATGG AGATGTTTCG GGAAGATGAA GGCTACATCA 400

AGGAGGAAGA GCGTCCTCTG CCTGAAAATG AGTTTCAGAG ACAAGTGTGG 450

CTTCTCTTTG AATACCCAGA GAGCTCAGGG CCTGCCAGGA TTATAGCTAT 500

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CATCACCCTG GGGACAGAGT TGGCTGAGAA GCCAGAGGAC GCTCAGCAAG 850

GCCAGCAGGC CATGTCACTG GCCATCCTCC GTGTCATCCG GTTGGTAAGA 900

GTCTTTAGGA TTTTCAAGTT GTCCAGACAC TCCAAAGGTC TCCAGATTCT 950

AGGTCAGACC CTCAAAGCCA GCATGAGAGA ATTGGGCCTC CTGATATTCT 1000

TTCTCTTCAT AGGGGTCATC CTTTTCTCTA GTGCTGTGTA TTTTGCAGAG 1050

GCCGATGAGC GAGAGTCCCA GTTCCCCAGC ATCCCAGATG CCTTCTGGTG 1100

GGCAGTCGTC TCCATGACAA CTGTAGGCTA TGGAGACATG GTTCCGACTA 1150

CCATTGGGGG AAAGATAGTG GGTTCCCTAT GTGCGATTGC AGGTGTGTTA 1200

ACTATTGCCT TACCGGTCCC TGTCATTGTG TCCAATTTCA ACTACTTCTA 1250

CCACCGGGAG ACAGAGGGAG AGGAGCAGGC CCAATACTTG CAAGTGACAA 1300

GCTGTCCAAA GATCCCATCC TCCCCTGACC TAAAGAAAAG TAGAAGTGCC 1350

TCTACCATTA GTAAGTCTGA TTACATGGAG ATCCAGGAGG GTGTAAATAA 1400

CAGTAATGAG GACTTTAGAG AGGAAAACTT GAAAACAGCC AACTGTACCT 1450

TGGCTAACAC AAACTATGTG AATATTACCA AAATGTTAAC TGATGTCTGA 1500

Appendix 3

HuKII and huKIV subunits efficiently assemble into heteromultimeric potassium channels with distinct physiological properties.

Introduction:

There is considerable evidence that voltage-dependent potassium channels form by the association of several subunits. The polypeptide encoded by a potassium channel gene is similar to a single homology domain of a sodium channel; this comprises only one fourth of the sodium channel protein. The similarities in the function of the two channel types and the relatedness of the sequences argue strongly that voltage-gated potassium channels are tetramers of small subunits, each subunit analogous to a sodium-channel homology domain. In addition, there is genetic evidence in *Drosophila* that shows that defective subunits may antagonize the function of wild-type subunits in the manner expected for a multimeric protein.

Many cloned potassium channel genes independently express functional channels in a variety of expression systems. These channels are presumably homomultimeric complexes of identical subunits. Eight distinct, homomultimeric channels are generated at the *Shaker* locus of *Drosophila* from alternatively spliced mRNAs. In vertebrates, at least 4 different potassium channel genes express distinct homomultimeric potassium channels. An exciting possibility is the formation of heteromultimeric channels, each with different properties. This would result in a potentially enormous number of potassium channels generated by subunit shuffling. The work presented in this appendix shows that heteromultimeric potassium channels form efficiently in *Xenopus* oocytes. The *in vivo* formation of such channel complexes remains to be demonstrated.

Results and Discussion:

Xenopus oocytes were coinjected with cRNA from huKII and huKIV cDNA; control oocytes were injected with individual RNAs. Voltage-dependent, potassium-selective currents were recorded by two microelectrode voltage clamp, in the manner described in Chapter 3. Currents observed in coinjected oocytes had a transient component

and a sustained component, apparently similar to an algebraic sum of individual huKII (transient) and huKIV (sustained) currents. However, while huKII currents are almost completely inactivated by a 1s prepulse at -40mV, the transient component in coinjected oocytes was not affected by this prepulse. Thus the transient component seen in coinjected oocytes differs from huKII in its voltage dependence of inactivation, and differs from huKIV in its rate of inactivation. This novel channel type is most likely to be a heteromultimeric species containing some huKII and some huKIV subunits. We also investigated the nature of the sustained component of the potassium current in coinjected oocytes. As this was clearly not huKII, which is transient in nature, we specifically tried to separate this component from huKIV currents. While huKIV currents are completely eliminated by 40nM charybdotoxin, a large fraction of the sustained component was not affected by this concentration of CTX. Thus, this CTX-insensitive, sustained potassium current is different from huKII and huKIV and is also likely to be a heteromultimeric species of potassium channel. These data are shown in Fig 1.

It is not yet completely clear whether the transient and sustained components represent two different heteromultimeric species or different components of the same heteromultimeric channel. We are attempting to resolve this issue by a more detailed physiological and pharmacological characterizaton of these currents. Interestingly, homomultimeric huKII and huKIV channels do not form a very large fraction of the currents that we seen in coinjected oocytes. This suggests that the heteromultimeric chanels form with comparable efficiency.

The potential diversity of voltage-gated potassium channels has been greatly increased by the identification of heteromultimeric channels. Eukaryotic cells may regulate the process of subunit assembly in some way to generate the specific oligomeric species that serve the cell's immediate requirements. The identification and analyses of such potential regulatory mechanisms promises to be a subject of much interest; especially

because these mechanisms may also operate on a wide range of molecules that include transcription factors, metabolic enzymes and membrane proteins.

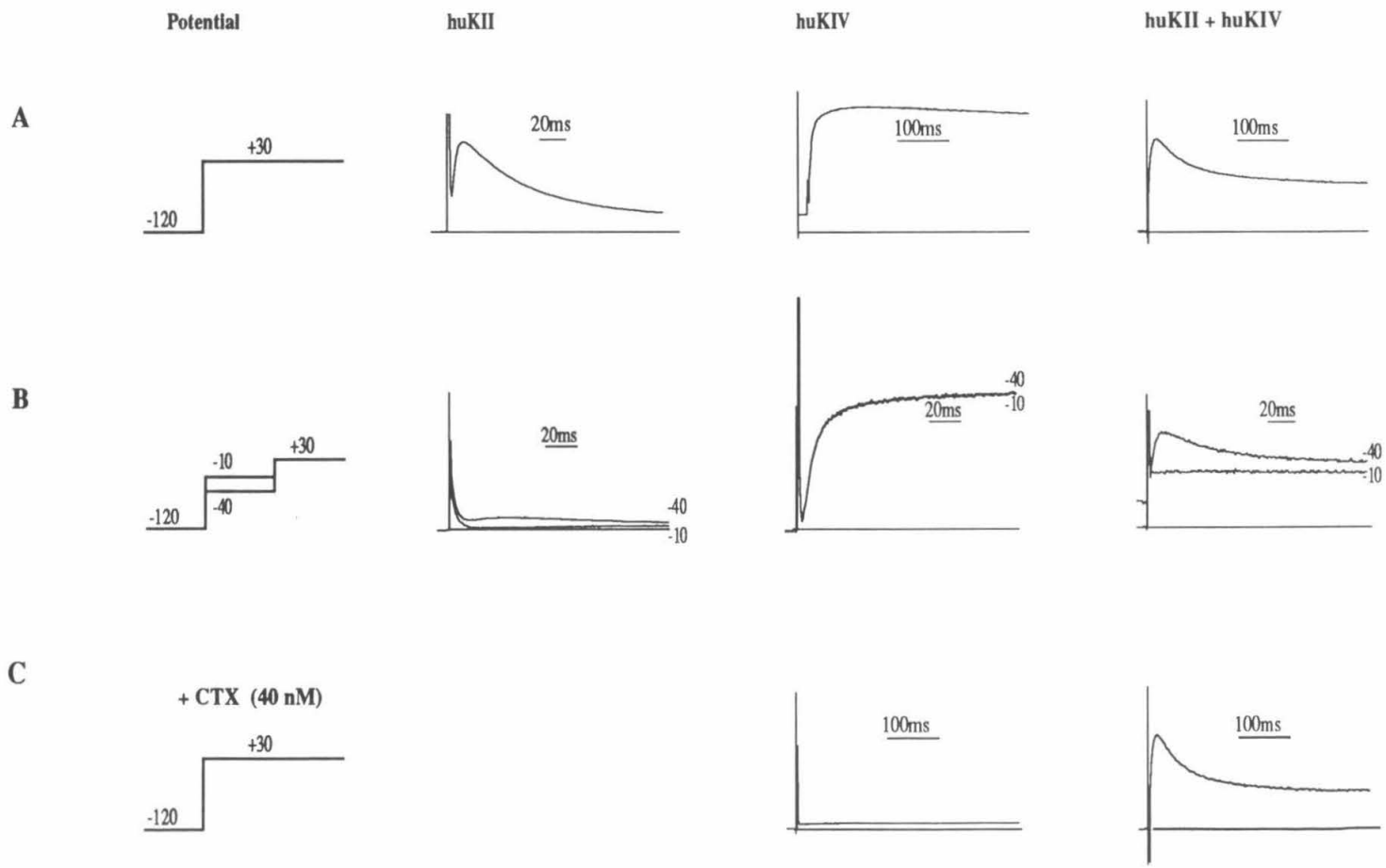
Fig. 1. Heteromultimers of huKII and huKIV:

Responses of *Xenopus* oocytes injected with huKII cRNA (column ii), huKIV cRNA (column iii), and huKII and huKIV cRNAs (column iv), to depolarizing voltage pulses (shown in column i).

A. Responses to depolarizations to +30 mV following a prepulse to -120mV for 2s, for channels to recover from inactivation. The transient component of current seen in coinjected oocytes inactivates with a time scale slightly different from huKII; however currents seen in coinjected oocytes may be roughly approximated by an algebraic sum of huKII and huKIV currents.

B. Responses to depolarization to +30mV following a prepulses for 1s at -40mV and -10mV. The -40 prepulse eliminates huKII currents but has little effect on the transient component in the coinjected oocytes. The prepulse at -10mV eliminates all transient currents, but has little effect on huKIV or the sustained component in coinjected oocytes. The voltage dependence of inactivation, of the transient component in coinjected oocytes, is shifted about 20mV positive to huKII (data not shown).

C. Responses, in the presence of 40nM charybdotoxin, to depolarizations to +30mV following a 2s prepulse at -120mV. HuKIV currents are completely eliminated by 40nM CTX, while currents in coinjected oocytes are not. It is possible that CTX marginally reduces currents in the coinjected oocytes but this reduction has not been analyzed in any detail.


Potential
huKII
huKIV
huKII + huKIV
A
+30
-120
20ms
100ms
100ms
B
-10
+30
-120
-40
20ms
-40
-10
-40
20ms
-10
20ms
-40
-10
C
+ CTX (40 nM)
+30
-120
100ms
100ms