STRUCTURE AND FUNCTION STUDIES OF A RAT BRAIN VOLTAGE-DEPENDENT POTASSIUM CHANNEL

by

Raymond S. Hurst

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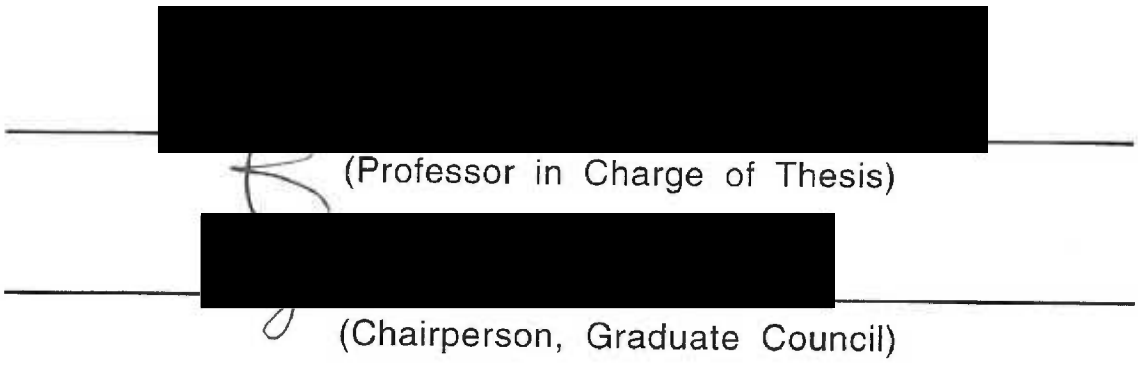

(Professor in Charge of Thesis)

(Chairperson, Graduate Council)

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ABSTRACT

This dissertation is concerned with the structure and functional mechanisms of a cloned rat brain voltage-dependent potassium channel, RBK1 (Christie, et al., 1989; also called Kv1.1). When expressed in *Xenopus* oocytes, this channel gives rise to a voltage-dependent K^+ current which activates rapidly upon membrane depolarization, and slowly inactivates over prolonged depolarizations. Potassium currents with these properties are found in some excitable cells with short action potentials where they function to keep the action potential brief (Hille, 1992).

The major questions regarding channel structure and function addressed by this research are as follows. **1.** Can specific amino acid residues be identified which contribute to the external mouth of the conduction pathway? **2.** What is the stoichiometry and symmetry of the outer channel mouth? **3.** Do channel subunits interact during the voltage-dependent opening of the pore? And **4.** can channels with radical amino acid substitutions in the putative voltage-sensor respond to membrane potential? To address these questions, a combination of molecular genetic and electrophysiological approaches were used. Briefly, mutant forms of RBK1 were generated and *in vitro* transcribed RNA was injected into *Xenopus laevis* oocytes. Channel function was assayed by measuring whole cell ionic currents, primarily using the two-electrode voltage-clamp technique. Some aspects of this research benefited from the development of a concatenated potassium channel ([4]RBK1) comprised of four linked RBK1 subunits. This construct

allowed for the expression of channels having a defined combination of different subunits (domains).

The channel blockers α-dendrotoxin (DTX) and tetraethylammonium (TEA) were used to probe the structure of the outer channel mouth. Identification of amino acids which influence the DTX potency helped locate the region of the channel protein which contributes to the outer pore. Sequential mutations introduced into [4]RBK1, one into each domain, that decreased TEA sensitivity provided the most direct evidence that the TEA binding site, and presumably the outer pore, are made by equal contributions of four subunits. Concatenated channel constructs were also used to test aspects of voltage-dependent activation and the putative voltage sensor (S4). A conservative amino acid substitution which alters activation voltage-dependence was introduced sequentially into the S4 segments of [4]RBK1. The activation curves of these constructs suggest that S4 segments interact cooperatively during the voltage-dependent opening of the channel pore. [4]RBK1 was also used to test the effect on activation of more radical mutations (*i.e.* a Pro in the putatively α-helical S4 segment) which destroy current expression when introduced into all four S4 segments.

INTRODUCTION

Ion transport, a brief overview.

Ion channels are integral membrane proteins which permit the passage of small inorganic ions across biological membranes, an otherwise excellent electrical insulator (Hille, 1992; Levitan and Kaczmarek, 1991). Ion channels can be thought of as enzymes which catalyze the transmembrane flow of physiological ions. Like enzymes, ion channels show substrate specificity, *i.e.* they allow passage of some ions while excluding others. In addition, ion channel activity, like that of enzymes, can be modulated by environmental cues, increasing or decreasing the flux of ions across the membrane. By regulating transmembrane ion flow, ion channels participate in diverse physiological process including excitation, secretion, motility, cell volume regulation, and possibly learning and memory (Hille, 1992; for review, see Jan and Jan, 1989; Catterall, 1988).

There are six major classes of ion channels: gap junctions, ligand gated channels, voltage-sensitive cation channels, voltage-sensitive anion channels, second messenger gated channels, and stretch-sensitive channels (for review, see Jan and Jan, 1989). This classification system is largely based on the ion selectivity of the channel and the cues which modify its activity. The classification is not absolute and there is considerable overlap. For example, the activity of ligand-gated and second messenger-gated channels can also be modulated by membrane potential. In addition, the activity of many voltage-gated ion channels can be modulated by second

messengers, cyclic nucleotides and G proteins (*e.g.* Ashcroft, 1988; Boyle et al., 1987; Levitan, 1988; Meyer et al., 1988; Piomelli et al., 1987; for review see Jan and Jan, 1989).

The commonalty of all ion channels is an aqueous transmembrane pore through which ions can diffuse. Ion flux through channels is driven by the electrochemical gradient of the ion. The electrochemical gradient, described by the Nernst equation (Nernst, 1888), is a function of the concentration ratio and valence of the permeant ion. According to this equation the equilibrium potential, *i.e.* the potential at which there is no net ionic flux, also called the reversal potential (E_{rev}), is defined as

$$E_{rev} = E_i - E_o = \frac{RT}{zF} ln \frac{[S]_o}{[S]_i} \qquad (1)$$

where $E_i - E_o$ is the potential difference across the membrane ($_i$ and $_o$ signify inside and outside, respectively), z is the ion's valence, [S] is the molar concentration of ion S, and R, T, and F have their usual meaning (see Hille, 1992). As can be seen above, the reversal potential E_{rev} varies logarithmically with the ion concentration ratio.

The electrochemical gradient is generated by the active transport of ions across the cell membrane. This process is catalyzed by pumps and transporters in the membrane which either directly or indirectly consume metabolic energy to move ions against their electrochemical gradient (Aidley, 1989). The primary active transport system in neurons, and most other animal cells, is

the Na^+-K^+ ATPase. Every cycle of this pump, which consumes one molecule of ATP, internalizes two K^+ ions and extrudes three Na^+ ions, developing a concentration gradient for both ions (Post and Jolly, 1957). Active transport systems have been described for many physiological ions including Ca^{2+}, Cl^-, H^+, and HCO_3^- (Aidley, 1989). Other cellular roles of active transport systems include the regulation of cell volume and pH, and metabolite uptake.

Ion channels are dynamic molecules which rapidly inter-convert between conducting and non-conducting states. This process, called gating, appears to be the opening and closing of the channel pore (Hille, 1992). The gating behavior of most channels can be modulated by some stimulus (*e.g.* membrane potential, neurotransmitters, second messengers, etc.). When the pore of the channel is in the conducting or open state, some ion species will flow across the cell membrane, down the electrochemical gradient, while other ion species are largely excluded. Gating behavior and ion selectivity underlie the cellular function of ion channels. Often, it is the combined efforts of different channel types that control membrane events related to ion flux. For example, the concerted regulation of Na^+, K^+, Ca^{2+}, and Cl^- permeability underlie excitation and electrical signaling in the nervous system (see Hille, 1992; Levitan and Kaczmarek, 1991; Aidley, 1989).

Potassium channels.

Potassium channels are by far the most ubiquitous of all ion channel families. They are found in almost every Eukaryotic cell type (Latorre and Miller, 1983; Hille 1984; Rudy 1988), including

plants (Schroeder et al. 1987; Iijima and Hagiwara 1987), fungi (Saimi et al. 1988) and algae (Bertl and Gradmann, 1987; for review, see Jan and Jan 1989). This channel family is also the most diversified. The various members can be distinguished on the basis of gating behavior, kinetics, pharmacology, single-channel conductance and other properties (for review, see Rudy, 1988). Potassium currents play important functional roles in both excitable and non-excitable cells. Cellular roles of K^+ currents include the regulation of cell volume, neuronal excitability, pace of the heart beat, and endocrine and exocrine secretion (for review, see Catterall, 1987).

To function in a biologically useful way, K^+ channels must perform two rather sophisticated tasks. They must rapidly switch between conducting and non-conducting conformational states in response to some environmental stimulus, and they must faithfully discriminate K^+ from other physiological ions such as Na^+, Mg^{2+}, Ca^{2+} and Cl^- (Miller, 1991). In excitable cells, there are three major sub-families of potassium channels, distinguished by their gating behavior: voltage-dependent K^+ channels, Ca^{2+}-dependent K^+ channels, and inward rectifying K^+ channels, all of which are selective for K^+.

The voltage-dependent K^+ channels underlie a major source of K^+ current in many types of cells (for review, see Rudy, 1988). Gating of these channels is modulated by the potential difference across the membrane; conductance increases with depolarization and decreases with hyperpolarization. In excitable cells, the function of voltage-dependent K^+ channels is electrical in nature. A potassium

ion carries one positive elementary charge (e) or 1.602×10^{-19} Coulombs. Consequently, a net flux of K^+ will change the potential difference across the cell membrane. The rate of K^+ flow through voltage-dependent K^+ channels typically ranges between 10^6 and 10^8 ions/sec (Yellen, 1987). Potassium flux across most cell membranes can measurably change the membrane potential quasi-instantaneously. Potassium, concentrated inside the cell, flows down its electrochemical gradient to the outside of the cell. This removes a net positive charge from the cytoplasmic side of the membrane, drawing it away from the firing threshold and decreases cell excitability (Hille, 1992). Potential dependent changes in K^+ permeability modulate action potential duration, time of the interspike interval, and terminate periods of intense activity (Hille, 1992).

The voltage-dependent K^+ channel family can be subdivided into two major categories based on kinetic behavior: delayed rectifiers and A-type channels (Hille, 1992; for review see Rudy, 1988). Delayed rectifiers, first described in 1952 by Hodgkin and Huxley (1952a), activate upon the depolarization of the membrane potential, following an initial delay. Delayed rectifiers are not a unique type of channel but rather a class of functionally related channels (Hille, 1992). For example, delayed rectifiers found in excitable cells with short action potentials activate rapidly, within milliseconds, following membrane depolarization. These channels are prevalent in unmyelinated axons, motoneurons, and vertebrate fast skeletal muscle. Other delayed rectifiers, such as those found

in heart and uterus, have activation kinetics that are orders of magnitudes slower (Hille, 1992).

A-type channels, like the delayed rectifiers, respond to membrane potential. They activate with membrane depolarization but not hyperpolarization, and the onset of the current follows an initial delay. A-type channels differ from delayed rectifiers in that they inactivate rapidly, in tens of milliseconds, during a maintained depolarization. These currents, originally described by Conner and Stevens (1971), are found in encoding membranes such as nerve cell bodies or sensory terminals where the action potential firing rate reflects the stimulus intensity. In these membranes, A-type channels space successive action potentials by acting as a damper in the interspike interval (Hille, 1992). This activity maintains action potential firing at a rate so as not to exhaust the nerves, muscles or glands that follow.

The preceding sections provide only a brief and rather incomplete description of K^+ channel function and diversity. Many other types of K^+ selective channels with unique properties and functions have been characterized. For example, a voltage-dependent K^+ channel of T lymphocytes is thought to be involved in mitogen-induced cell proliferation and cytotoxic killing (Cahalan et al., 1985; DeCoursey et al., 1984; Chandy et al., 1984, Schlichter et al., 1986). The K^+ channel family also includes members which respond to diverse stimuli, including stretch (Eckert and Brehm, 1979) and light (Gorman et al., 1982). A complete description of K^+ channel function and diversity has been covered in several excellent

textbooks and reviews (*e.g.* Hille, 1992; Aidley, 1989; Levitan and Kaczmarek, 1991; Rudy, 1988; Jan and Jan, 1989).

Theory and methods for studying ionic currents.

The voltage-clamp technique, developed by Marmont (1949), Cole (1949), and Hodgkin, Huxley, and Katz (1949,1952), has proven to be the best means for studying currents passing through ion channels. The principal of this technique is to hold constant or "clamp" the membrane potential and record the transmembrane current. In its simplest form, the voltage clamp consists of two intracellular electrodes; one measures the potential near the membrane and the other passes current (see Hille, 1992; Levitan and Kaczmarek, 1991). The membrane potential (E_m) is constantly measured and compared to the command potential ($E_{command}$) set by the experimenter. A feedback amplifier injects whatever current is required to minimize the difference between E_m and $E_{command}$. The injected current, which passes between the intracellular current electrode and a ground electrode in the extracellular solution, is equal to the net membrane current (I_m). I_m is comprised of two components; the ionic current (I_i), which results from ions crossing the membrane, and the capacity current (I_C), which results from ions moving up to the membrane to charge or discharge its capacitance (Hille, 1992).

$$I_m = I_i + I_C \qquad (2)$$

Except at the moment when the membrane potential is jumped from one level to another, the change in membrane potential, dE/dt, is zero and I_C stops flowing. In this case (*i.e.* voltage clamp), I_m is equal to the ionic component I_i. Much of what is know about ion channels and their function comes from voltage-clamp recordings of I_i.

The amount of current i that will pass through any given single channel is a function of the channel's conductance (γ) and the driving force ($E_{rev} - E_m$) on the ion, where E_m is the potential difference across the membrane.

$$i = \gamma \cdot (E_{rev} - E_m) \qquad (3)$$

This equation is simply Ohms law where the conductance γ, measured in Siemens, is the reciprocal of electrical resistance R. The total current passing across the membrane at any instant in time is the sum of the current passing through all channels in the membrane that are open at that moment. Because the process of channel opening and closing is stochastic (see Colquhoun and Hawkes, 1977, 1981), the number of open channels in a membrane is constantly changing. Consequently, the time-averaged current passing through a given channel species is the product of the number of channels in the membrane (N), the probability that a channel is in a conducting or open conformation (p_o), and the unitary current i.

$$I_m = N \cdot p_o \cdot i \qquad (4)$$

Measurements of I_m can provide information on the activity of the channels. For example, by holding the driving force (E_m - E_{rev}) constant in voltage-clamp experiments, i will remain constant and channel activity can be assessed under different conditions (*e.g.* drug concentration and membrane potential).

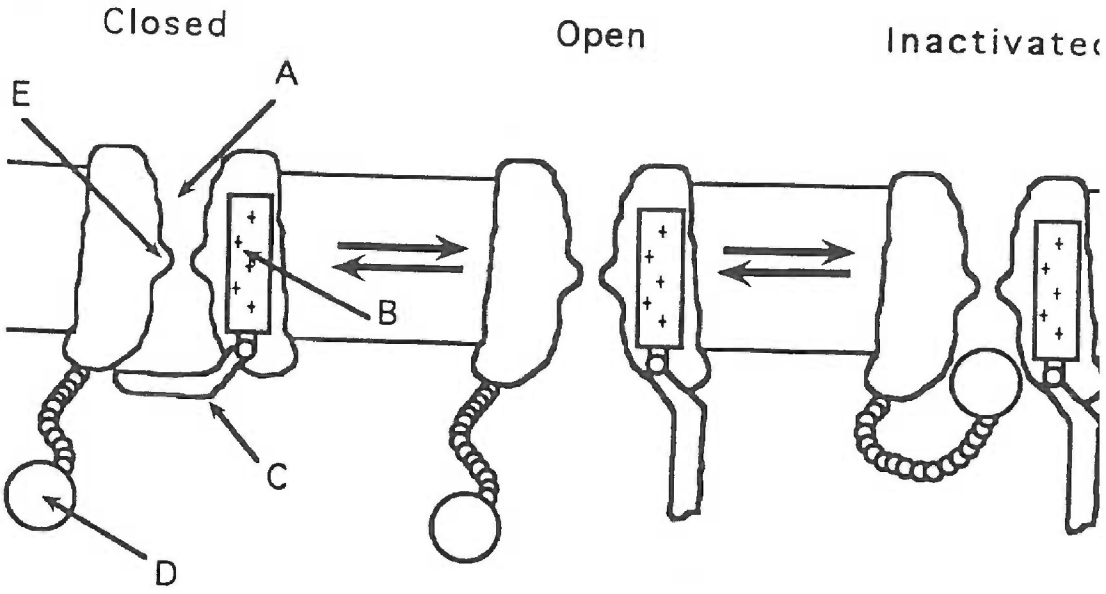
Native membranes often express many different channel types and consequently I_m is comprised of several components, one for each channel type. Overlapping channel expression generally complicates the study of ion channels. One approach to simplify these measurements has been to eliminate all but the current of interest using specific blockers and ionic conditions. This method has been extremely productive in some preparations but is often a formidable task. The relatively recent cloning of ion channels has greatly simplified this process. Cloned ion channels are commonly expressed in an otherwise electrically silent cell. In this case, I_m results solely from the channel species of interest. Oocytes isolated from the African clawed frog *Xenopus laevis* have proven exceptionally amenable to this procedure. *In vitro* transcribed RNA encoding the cloned channel is injected into the oocyte; the message is translated, and the product is assembled and inserted into the membrane. The combined high expression of the injected RNA and the low density of endogenous channels often allow for measurements of essentially homogenous ionic currents. The function of most cloned ion channels have been assayed using this or a similar method.

Topology and functional domains of voltage-dependent potassium channels.

Functionally, voltage-dependent K^+ channels are comprised of up to five major domains: an aqueous transmembrane pore, a selectivity filter, a voltage sensor, an activation gate, and in the case of A-type channels an inactivation domain (Fig. 1). The relatively low density of K^+ channels (mean < $1/\mu m^2$; *e.g.* Rehm and Lazdunski, 1988; Schmidt and Betz, 1988) combined with their large size and hydrophobic nature has hindered more traditional approaches to study protein structure. Essentially all that is known about the structure and functional domains of K^+ channels comes from the combined approaches of biochemistry, electrical measurements, and molecular genetic techniques.

Structure and function studies of voltage-dependent K^+ channels were initiated by the cloning of the *Shaker* locus of *Drosophila*. *Shaker* mutants have long been known to demonstrate general hyperexcitability and shake their legs vigorously while anesthetized. This phenotype is related to an altered or missing A-type current in some muscles and neurons (Jan et al., 1977; Salkoff and Wyman, 1981). This mutation was localized on polytene chromosomes (Tanouye, 1981) and subsequently cloned by chromosome walking (Papazian et al., 1987; Kamb et al., 1987; Baumann et al., 1987). The single gene encodes at least four different translatable products via alternative splicing (Tempel et al., 1987; Schwarz et al., 1988; Pongs et al., 1988; Kamb et al., 1988). *In vitro* transcribed RNA encoding each variant gives rise to a distinct voltage-dependent K^+ current when injected into *Xenopus*

Figure 1.



A. Aqueous transmembrane pore
B. Voltage-sensor
C. Activation gate
D. Inactivation domain
E. Selectivity filter

oocytes (Iverson et al., 1988; Timpe et al., 1988a; Timpe et al., 1988b).

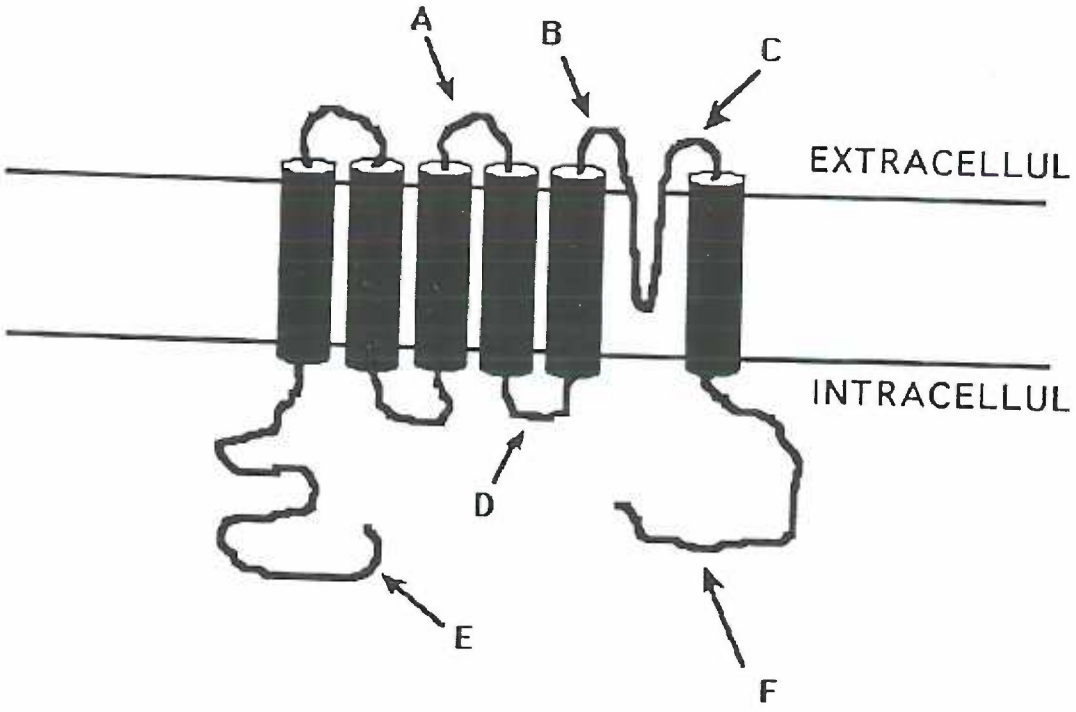
The predicted amino acid sequence of the *Shaker* gene revealed two prominent structural features of this channel family (Tempel et al., 1987). First, hydropathy plots identified six or seven stretches of amino acids sufficient in length and hydrophobic moment to span the membrane. Second, the fourth putative membrane spanning domain (the S4 segment) contained a repeated motif of basic residues separated by two hydrophobic residues (- Lys/Arg - Xxx - Xxx -, where Xxx represents a hydrophobic residue) (Tempel et al., 1987). This is the only segment of amino acids showing sequence homology to the eel Na^+ channel and was postulated to be involved in the voltage dependence of channel activation (Greenblat et al., 1985; Guy and Seetharamulu, 1986; Noda et. al., 1986; Catterall, 1986).

Initiated by the cloning of *Shaker*, a long and growing list of invertebrate and vertebrate cDNAs encoding voltage-dependent K^+ channels have been isolated (Hille, 1992; Levitan and Kazmarek, 1991; for review, see Jan and Jan, 1992). A total of four potassium channel genes have been identified in *Drosophila*: *Shaker*, *Shab*, *Shaw* and *Shal*. When expressed in oocytes, each gives rise to a kinetically distinct voltage-dependent K^+ current (Wei et al., 1990). Mammalian homologues of each *Drosophila* K^+ channel gene have been identified and expressed. The extensive repertoire of cloned voltage-dependent K^+ channels has benefited the study of channel structure and function. Comparisons of the predicted amino acid sequence between channels with different properties can provide insight to the location of functional domains (*i.e.* the region of the channel

protein providing a function). Using this approach, Guy and co-workers have developed computer models of ion channel structure (Guy and Seetharamulu, 1986; Guy and Conti, 1990; Durell and Guy, 1992). Models of channel structure and function are constantly being tested experimentally by the combined efforts of many different groups using electrophysiological, biochemical, and molecular genetic approaches. A complete description of these studies is beyond the scope of this dissertation; the following is meant only to cover the salient features.

1. Membrane topology. It is now generally believed that voltage-dependent K^+ channel subunits of the *Shaker* family cross the membrane six times (S1 through S6), with intracellular amino and carboxyl termini (Fig. 2.; see Guy and Conti, 1990; Durell and Guy, 1992, for review, see Pongs, 1992). This topology was first suggested by hydropathy plots and the observation that the amino terminus of channel protein lacked a recognizable signal sequence (Tempel et al., 1987). Experimental evidence consistent with this topology has been provided along three lines. First, specific amino acid residues in the loop between the fifth (S5) and sixth (S6) putative membrane spanning domains have been identified which influence the potency of the extracellular channel blockers charybdotoxin (CTX) (MacKinnon and Miller, 1989), tetraethylammonium (TEA) (Kavanaugh et al., 1991; MacKinnon and Yellen, 1990) and dendrotoxin (DTX) (Hurst et al., 1991). Together, these results provide strong evidence that residues between S5 and S6 reside outside the cell.

Figure 2.



A. Extracellular -- Antibody-binding (Chua et al., 1992).

B. Extracellular -- Point mutations influence potency of CTX (MacKinnon and Miller, 1989), TEA (MacKinnon and Yeller, 1990; Kavanaugh et al., 1991), and DTX (Hu et al., 1991).

C. Extracellular -- Point mutations influence potency of TEA (MacKinnon and Yeller, 1990; Kavanaugh, 1991) and DTX (Hurst et al., 1991).

D. Intracellular -- Point mutations influence inactivation kinetics (Isacoff et al., 19

E. Intracellular -- Deletions abolish rapid inactivation (Hoshi et al., 1990).

F. Intracellular -- Antibody-binding (see Pongs, 1988).

Second, antibody-binding studies have identified specific regions as intracellular or extracellular. An antigenic sequence wa inserted between the third (S3) and fourth (S4) putative membrane spanning domains of RCK1 (Chua et al., 1992; also called Kv1.1). Native oocytes expressing these channels were stained with a monoclonal antibody against the epitope, localizing this region to the extracellular surface of the membrane. Similarly, antibody-binding studies have reportedly localized the carboxyl terminus to the intracellular surface of the membrane (see Pongs, 1988).

Finally, the amino terminus and the S4-S5 loop have been localized to the cytoplasmic face of the membrane based on their involvement in fast inactivation (see below). Mild intracellular treatment of rapidly inactivating *Shaker* K^+ channels with protease abolishes inactivation (Hoshi, et al., 1990; see also Demo and Yellen, 1991; Armstrong et al., 1973). Site-directed mutagenesis and deletion experiments have localized the affected region, called the inactivation domain (see Fig. 1.), to amino terminus of *Shaker* B (*Sh*B), implying it resides intracellularly (Hoshi et al, 1990; Zagotta et al., 1990). Likewise, specific amino acid substitutions in the loop between S4 and S5 of *Sh*B alter the kinetics of rapid inactivation (Isacoff et al., 1991). This region is thought to act as the receptor site for the inactivation domain, suggesting it also resides on the cytoplasmic side of the membrane.

2. Permeation pathway. A major goal of molecular studies on K^+ channels is to identify the region of the channel protein which forms the lining to the conduction pathway. The aqueous pore through which K^+ passes is in fact a highly specialized structure.

When open, this structure must allow K^+ to freely pass, at rates which can approach diffusion limitations (for review, see Yellen, 1987), yet exclude other physiological ions such as Na^+, $\approx$ 0.4 Å smaller in radius (Pauling, 1960). Ion selectivity is of fundamental importance for electrical excitability; without it, electromotive forces could not be generated (Hille, 1992).

A wealth of evidence suggests that the permeation pathway is comprised of amino acids in the loop between S5 and S6. Localization of the CTX binding site to the S5-S6 loop initially suggested this region contributed to the outer channel mouth (MacKinnon and Miller, 1989). CTX is thought to block K^+ conduction by physically occluding the pore (Anderson et al., 1988); therefore, residues which influence CTX potency should reside near the pore. Similarly, residues which interact with the external channel blocking compounds TEA and DTX, also thought to block conduction by occluding the permeation pathway, have been identified in the S5-S6 loop (MacKinnon and Yellen, 1990; Kavanaugh et al., 1991; Hurst et al., 1991). Together, these studies provide good evidence that amino acids between S5 and S6 contribute to the outer mouth of the permeation pathway (Fig. 2.). The identified amino acids are located in two clusters, approximately 21 residues apart.

Three lines of experiments, performed independently, strongly suggest that the S5-S6 loop spans a major portion of the membrane and forms the lining of the K^+ conducting pore. First, Yellen and co-workers identified a Thr residue that influenced the ability of internally applied TEA to block potassium conduction (1991). This position (Thr^{441} in *Shaker*) is centered between the two clusters of

amino acids that influence external TEA, CTX, and DTX binding. This implies that the 21 amino acid stretch between the external binding sites folds across a significant portion of the membrane such that Thr^{441} can be accessed cytoplasmically. Second, Yool and Schwarz made amino acid substitutions in the S5-S6 loop of *Sh*A which enhanced the conduction of NH_4^+ and Rb^+ (1991). This suggests that the targeted residues (Tyr^{422}, Thr^{441}, Thr^{442}) are likely to reside within the conduction pathway. Finally, Hartmann and co-workers removed a 21 amino acid segment from the S5-S6 loop of one channel (NGK2) and transplanted it into a second channel (DRK1) having different conduction properties (1991). The chimeric channel (DRK-NGK) had conduction properties similar to the donor channel NGK2, suggesting that this 21 amino acid stretch comprises a major portion of the conduction pathway. Taken together, these results provide convincing evidence that the pore forming region of voltage-dependent K^+ channels is located between the fifth and sixth putative membrane spanning domains. This region is now referred to as the (P) or pore forming region (Stevens, 1991).

3. Voltage-dependent gating. In their original description of the ionic conductance in the squid giant axon, Hodgkin and Huxley suggested that the voltage-dependence of ionic conductance was dependent on the movement of charged particles across the membrane electric field (1952b). Movement of this charge (*i.e.* the voltage sensor, see Fig. 1.) across the membrane field would be expected to generate current. Successful measurement of such "gating currents" was first accomplished by Armstrong and Bezanilla (1973, 1974) and Keynes and Rojas (1974). The S4 segment, found in

all cloned voltage-dependent ion channels, is well suited to play the role of the voltage-sensor. It contains a series of charged residues and is predicted to span the membrane. This region has been the focus of numerous site-directed mutagenesis studies, supporting its involvement in the voltage-dependent opening of the channel pore. Amino acid substitutions for both charged (Papazian et al., 1991; Logothetis, et al., 1992; Liman et al., 1991; Tytgat and Hess, 1992) and hydrophobic (Lopez, et al., 1991; McCormack et al., 1991; Hurst et al., 1992) residues alter the voltage-dependence of channel activation. While it is clear from these experiments that the S4 region is involved in the voltage dependent operation of these channels, little is known about the precise nature of this mechanism. For example, Logothetis and co-workers showed that neutralization of the positive charges at positions 1 and 7 (numbers indicate the position of charge within the S4 segment of Kv1.1, see Logothetis et al., 1992) gave rise to channels that required more positive depolarizations to become activated, but neutralization at position 2 produced channels that activated at less depolarized potentials (1992). These results suggest that the S4 segment does not act as a homogenous charged structure, but that individual residues may have specific roles in channel activation.

The functional domain responsible for the open $\rightleftarrows$ closed transition (*i.e.* the activation gate, see Fig. 1.) has not been localized; however, biophysical studies have provided some clues. In a classical study on the interaction of quaternary ammonium (QA) compounds with K^+ channels, Armstrong localized the activation gate to the cytoplasmic $\approx$ 20% of the membrane electric field (1966,

1969, 1971). TEA and higher affinity long chain derivatives of TEA were injected into the axoplasm of squid giant axons. Block by high affinity TEA derivatives lagged behind the current onset following a step depolarization. The time dependence of current inhibition suggests that the receptor site for these compounds is only available when the channel is conducting, *i.e.* when the activation gate is in the open conformation. Internally applied QA compounds block the channel pore by physically occluding the permeation pathway, and the receptor site is located approximately 20% across the electric field (for review, see Stanfield, 1983). This implies that the activation gate is located on the cytoplasmic side of the channel.

4. Inactivation. Inactivation is the process which closes voltage-dependent ion channels during a maintained depolarization (Hille, 1992). Once a channel becomes inactivated, the membrane must be repolarized or even hyperpolarized before channels are free to open again. Inactivation of voltage-gated K^+ channels can occur within milliseconds (A-type channels) or only incompletely over periods of seconds (delayed rectifiers). Long before the cloning of any voltage-dependent ion channel, Armstrong and Bezanilla conceptually described the mechanism of fast inactivation as the "ball and chain" model (1977). This model was based on the observation that trypsin treatment of the cytoplasmic face of Na^+ channels abolished current inactivation (Armstrong, Bezanilla, Rojas, 1973; Rojas and Rudy, 1976; Stimers, Bezanilla and Taylor, 1985). The "ball-and-chain" model proposed that a cytoplasmic region of the channel (the "ball", tethered to the rest of the channel

by a "chain") moves into the conduction pathway after the channel opens, blocking ion conduction. The channel domain responsible for fast inactivation has been localized to the amino terminus in *Shaker* channels (Hoshi et al., 1990; Zagotta et al., 1990). In these experiments, deletion of amino acids 6 - 46 in *Sh*B completely abolished rapid inactivation (Hoshi et al., 1990). Inactivation was restored by the addition of a synthetic peptide encoding those amino acids to the intracellular solution (Zagotta et al., 1990). Together, these studies provide convincing evidence that the amino terminus constitutes the blocking component of the fast inactivation mechanism.

Slow inactivation, observed in both A-type channels and delayed rectifiers, occurs over hundreds of milliseconds to seconds and is often incomplete. The mechanism for this type of current inactivation is not known. Slow inactivation is a distinct process from fast inactivation. For example, pronase (Rudy, 1978), N-bromoacetamide (Heggeness and Starkus, 1986), and papain (Quandt, 1987) all reduce the degree of fast inactivation in sodium channels but leave slow inactivation unaltered. The functional domain responsible for slow inactivation has not been localized to any specific region of the channel protein. Deletions of the amino and carboxyl terminus influence slow inactivation kinetics (Van Dongen et al., 1990). In addition, point mutations within S6 region (Hoshi et al., 1991) and the S5-S6 loop (Busch et al., 1991) alter the kinetics of slow inactivation. Proposed mechanisms for this form of inactivation include divalent cation binding (DeCoursey et al., 1984; Grissmer and Cahalan, 1989; Douglass et al, 1990; Busch, et al.,

1991) and slow voltage-dependent conformational changes (Ruben et al., 1992).

5. Subunit stoichiometry. The similarity in predicted membrane topology between any one of the four repeated domains in Na^+ and Ca^{2+} channels and the single domain in K^+ channels suggested the possibility that K^+ channels existed as multimers (Tempel et al., 1987). Genetic approaches have provided strong evidence that functional K^+ channels form as multimers. Mutant alleles of the *Shaker* gene do not make independent contributions to the A-type current when combined in heterozygotes (Timpe and Jan, 1987; Haugland and Wu, 1990; for review, see Aldrich, 1990). In addition, over-expression of a truncated *Shaker* protein in *Drosophila* interferes with the function of native A-type currents (Gisselmann et al., 1989). These experiments provide evidence that K^+ channels are formed as multimers in *Drosophila*. Further evidence for multimeric channels was provided by co-expression experiments. Briefly, two different K^+ channel encoding RNAs were injected into a single cell (Christie, et al., 1990; Isacoff et al., 1990; Ruppersberg et al., 1990). The resulting whole cell currents were assayed for properties that distinguished the two channel types when expressed individually. In each case the evoked ionic currents could not be described simply as the sum of the two different channel populations. These results can only be explained if novel channels were formed, having properties different from either channel when expressed alone. This provides strong evidence that channels can form by the heteropolymerization of different channel

subunits when expressed in the same cell (for review, see Aldrich, 1990).

The subunit stoichiometry of K^+ channels was first analyzed by MacKinnon (1991). Briefly, *Shaker* K^+ channel subunits that were either sensitive (wild-type) or insensitive (mutant, D431N) to block by extracellular CTX were co-expressed in the same cell. Statistical analysis of CTX dose response measurements indicated that the functional channels were much more likely to comprised of four subunits than three or five subunits. The statistical approach used by MacKinnon was based on two assumptions: **1.** subunit association was random and **2.** only channels comprised exclusively of mutant subunits (D431N) were weakly sensitive to CTX (MacKinnon, 1991). A more direct approach to test subunit stoichiometry has been to covalently join K^+ channel subunits at the DNA level (Liman et al., 1992; Hurst et al., 1992). Channel constructs comprised of 1, 2, 3, or 4 joined TEA sensitive domains were co-expressed with free TEA insensitive subunits.[1] Only the channel constructs comprised of four linked domains did not incorporate the free TEA-insensitive subunit. These experiments support the notion that channels are formed by the association of *four* subunits.

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[1] External TEA sensitivity can be reduced by a single amino acid substitution (Tyr→Val or Lys) at the outer mouth of the permeation pathway (MacKinnon and Yellen, 1990; Kavanaugh et al., 1991).

Biophysical properties of channel gating.

Like all chemical reactions, voltage-dependent K^+ channels undergo reversible transitions between all possible discrete chemical states (Colquhoun and Hawks, 1981). It is of interest to understand the properties of each transition because their aggregate describes the likelihood that a channel will be conducting at any given moment in time. The fundamental rules used to describe the biophysical mechanisms of ion channel function are based on electrostatics and kinetic theory (Hille, 1992). A major goal of the biophysical study of ion channels is to provide a kinetic description of channels which closely approximate the real behavior. These models can provide insight to the molecular mechanisms of ion channel function.

The simplest reaction scheme for a hypothetical ion channel consists of a single transition between an open (conducting) and closed (non-conducting) state

$$\text{Closed} \underset{\beta}{\overset{\alpha}{\rightleftharpoons}} \text{Open} \qquad (5)$$

where α and β represent the forward and backward rate constants, respectively. For voltage-gated channels, the transition rates between the open (O) and closed (C) state, and therefore the ratio O:C, are a function of the membrane potential. The voltage-dependence of the $C \rightleftarrows O$ conformational change arises from the movement of some net charge (z), called the gating charge, across the full potential drop E of the membrane (Hille, 1992). In energetic

terms, the ratio of opened to closed channels at equilibrium is described by the Boltzmann equation,

$$\frac{O}{C} = exp\left(-\frac{\omega - zeE}{kT}\right) \qquad (6)$$

where ω is the conformational energy increase upon the transition from C→O in the absence of a membrane potential (E=0), k is the Boltzmann constant, and e is the elementary charge (see Hille, 1992). The term $-zeE$ is the electrical energy increase for movement of the gating charge z across the membrane; the total change in free energy (ΔG) for this reaction is therefore (ω-zeE). Rearranging this equation gives the fraction of open channels, or open probability (p_o), as a function of the membrane potential E.

$$\frac{O}{O + C} = p_o = \frac{1}{1 + exp[(\omega - zeE) / kT]} \qquad (7)$$

According to reaction scheme (5), a new equilibrium between open and closed states is established with every membrane potential. The time course for settling to a new equilibrium upon a step change in membrane potential is dependent on the transition rate constants α and β. For the reaction scheme (5), the change in the concentration of open channels O as a function of time is described by the simple differential equation

$$\frac{dO}{dt} = \alpha(C) - \beta(O) \qquad (8)$$

Because the total number of channels (O + C) is constant, this can be re-written as

$$\frac{dO}{dt} = \frac{O_\infty - O_0}{\tau} \qquad (9)$$

where O_∞ is the steady state and O_0 is the initial value of O, and τ is the voltage dependent time constant defined as $1/(\alpha+\beta)$. The time course of O can be solved by integrating the differential equation above (9) giving

$$O_{(t)} = O_\infty - (O_\infty - O_0)\exp(-t/\tau) \qquad (10)$$

The fraction of open channels in (5) will therefore rise and fall exponentially following a step change in membrane potential. Membrane K^+ conductance on the other hand rises sigmoidally with time, following a step depolarization, and falls exponentially upon repolarization (Hille, 1992). Hodgkin and Huxley noted that such kinetics would be obtained if the opening of K^+ channels was controlled by four independent "gating particles", each of which must be in a permissive conformation for the channel to pass current (1952b). In physical terms, this can be described as four sequential voltage-dependent conformational changes, the last of which leads to a conducting state. The simplest reaction scheme to account for the observed kinetics of K^+ conductance is shown below.

$$\mathbf{C} \underset{\beta}{\overset{4\alpha}{\rightleftharpoons}} \mathbf{C} \underset{2\beta}{\overset{3\alpha}{\rightleftharpoons}} \mathbf{C} \underset{3\beta}{\overset{2\alpha}{\rightleftharpoons}} \mathbf{C} \underset{4\beta}{\overset{\alpha}{\rightleftharpoons}} \mathbf{O} \qquad (11)$$

It should be noted that this reaction scheme (11), the Hodgkin Huxley (HH) model, was developed prior to the molecular characterization of any voltage-dependent ion channel, indeed, before ion channels *per se* were known to exist, yet it can easily be correlated to the predicted four fold symmetry of these channels. That is, each transition can be thought to represent a voltage-dependent conformational change within an individual subunit, the open state being achieved after each subunit undergoes this conformational change. Kinetic models, such as the HH model, provide a means to contemplate the molecular mechanisms of ion channel function. While the details are not to be taken literally, these models can provide important mechanistic information. For example, the S-shaped activation time course of K^+ currents imply that several components or steps in a series control channel opening (Hille, 1992).

The HH model has proven remarkably useful for further biophysical characterization of ion channels. The development of new techniques and methods have resulted in some refinement of their original model. For example, the patch-clamp technique, developed by Neher and Sakmann, allows the measurement of single channel opening and closing events (1976). These measurements can provide substantial kinetic information on transitions between open and closed states. In the specific case of *Shaker*-like voltage-dependent K^+ channels, single channel analysis have provided strong evidence that the final transition into the open state is relatively independent of voltage (Zagotta and Aldrich, 1990; Koren et al.,

1990). More recently, improvements have been made in the oocyte recording system providing for high resolution measurements of intramembrane charge movement, *i.e.* gating currents (Bezanilla et al., 1991; Taglialatela et al., 1992; Taglialatela and Stefani, 1993). Measurements of gating currents can provide information on the transition between closed states, which impart the voltage-dependence on channel gating (Bezanilla et al., 1991; Taglialatela and Stefani, 1993). Finally, molecular genetic approaches (*e.g.* site-directed mutagenesis) have resulted in an explosion or information regarding the functional domains of voltage-dependent K^+ channels. These approaches are constantly being refined and improved, providing an even better understanding of channel structure and function.

Goals

The main objective of the work described in this dissertation was to characterize, in part, the structure and functional mechanisms of RBK1 (Kv1.1), a rat brain-derived, voltage-dependent K^+ channel of the *Shaker* family (Christie et al., 1989). This work was performed using both molecular genetic and electrophysiological techniques. Briefly, mutant forms of RBK1 were generated and expressed in *Xenopus* oocytes. Whole cell voltage-clamp measurements evoked from those cells were used to assay channel function. The first chapter describes a systematic approach to localize the binding site for DTX. These experiments helped identify the region of the channel protein which forms the outer channel mouth. The second chapter describes the development

and use of a concatenated K^+ channel to study channel structure and subunit interactions. Experiments in the first part of this chapter probe the structure of the outer channel mouth by characterizing the external TEA binding site. In the second part, cooperative interactions were tested by generating channels comprised of unique subunits which respond differently to membrane potential. The final chapter describes initial studies to probe in more detail the structure and functional mechanisms of the putative voltage-sensor.

Electrophysiology experiments generating data shown in some figures of the first and second chapters of this dissertation were performed by collaborators (see "Acknowledgments"). Mike Kavanaugh, Andy Busch, and Peregrine Osborne performed DTX concentration response measurements shown in Chapter 1. Mike Kavanaugh and Jerry Yakel performed most TEA concentration response measurements shown in Chapter 2.

CHAPTER 1

IDENTIFICATION OF AMINO ACID RESIDUES INVOLVED IN DENDROTOXIN BLOCK OF RAT VOLTAGE-DEPENDENT POTASSIUM CHANNELS

R. S. Hurst, A. E. Busch, M. P. Kavanaugh, P. B. Osborne, R. A. North, and J. P. Adelman

Summary

α-Dendrotoxin (DTX) is a 60-amino acid peptide belonging to the family of mamba snake neurotoxins; it is a potent blocker of some but not all voltage-dependent potassium currents. Potassium currents recorded from oocytes injected with cloned channel RNAs also vary in sensitivity to DTX. Expression of channels that were chimeras of the DTX-sensitive channel RBK2 and the DTX-insensitive channel RGK5 showed that the putative extracellular loop between transmembrane domains S5 and S6 contributes strongly to DTX sensitivity. Mutation of two residues (Ala^{352} Glu^{353}) in this region of RBK1 to conform to those at equivalent position in RGK5 (Pro^{374} Ser^{375}) reduced the potency of DTX about 70-fold, and the substitution of Tyr^{379} in RBK1 by its counterpart in RGK5 (His^{401}) caused an additional 2.5-fold decrease in sensitivity. Converse substitutions in RGK5 significantly increased sensitivity to DTX. The results suggest that these residues contribute significantly to the channel-toxin interaction, providing further evidence that the S5-S6 loop lies at or near the external mouth of the channel, where DTX binding leads to channel occlusion. They offer a molecular

explanation for the differences in DTX sensitivity observed among native potassium channels.

Introduction

DTX is one of a family of basic peptide neurotoxins isolated from mamba snake venom (Harvey and Anderson, 1985). Initially, mamba venom neurotoxins were shown to cause muscle paralysis, by either blockade of acetylcholine receptors or reduction in transmitter release (for review, see Chang, 1979 and Harris, 1984). In contrast, venom from one particular member of the family, *Dendroaspis angusticeps*, was found to enhance rather than block neuromuscular transmission. Harvey and co-workers (Harvey and Karlsson, 1980; Barrett and Harvey, 1979) demonstrated that the observed effects were due to increased acetylcholine release from nerve terminals. The increase of neurotransmitter release is now believed to result from block of voltage-dependent K^+ conductance, leading to action potential broadening. For example, peripheral administration of nanomolar concentrations of DTX induces repetitive firing in rat visceral sensory neurons by inhibiting a slowly inactivating outward K^+ current (Stansfield et al., 1986). Intracerebral administration at doses 10,000 times lower than the lethal peripheral dose induces convulsions and death; these actions have been ascribed to facilitation of transmitter release in the hippocampus (Docherty et al., 1983).

Dendrotoxins share significant homology with the Kunitz family of protease inhibitors. These peptides are believed to form stable, pear-shaped structures of about 3 x 2 nm. Most of the

molecule is thought to comprise a twisted antiparallel β-sheet with short α-helical regions at the amino and carboxyl termini. All members of the family are about 60 amino acids in length with six conserved cysteines, which might stabilize the structure through the formation of disulfide bonds (Harvey and Anderson, 1985). All carry net positive charges, due to up to nine positively charged residues. In particular, those toxins that facilitate transmitter release bear four conserved lysine residues. Because DTX blocks some but not all potassium conductances, it has been useful in the pharmacological classification of channels (Rudy, 1988; Moczydlowski et al., 1988; Castle et al., 1989). Its high affinity and selectivity also led to its use as a probe for channel isolation, and recently such a protein was purified from rat brain (Rehm et al., 1989).

The isolation and functional expression of cloned voltage-dependent potassium channels from *Drosophila* (Tempel et al., 1987; Wei et al., 1990; Papazian et al., 1987; Kamb et al., 1987) and mammals (Christie et al., 1989; Douglass et al., 1990; Stühmer et al., 1989; French et al., 1989; Swanson et al., 1990) offer a more direct approach to study the mechanism of action of DTX. The proteins encoded by the cloned cDNAs display significant primary sequence homology and have been predicted to cross the membrane six times, with both the amino and carboxyl termini residing intracellularly (Guy and Conti, 1990); the functional channel is believed to be a multimer, probably comprising four subunits, which may be either the same or different (Christie et al., 1990; Isacoff et al, 1990; Ruppersberg et al., 1990). The variation in DTX sensitivity

exhibited by the cloned and expressed potassium channels provides the opportunity to correlate primary sequence with DTX sensitivity and thereby determine regions of the channel involved in toxin binding. The present paper reports experiments in which such studies were carried out by expressing channel proteins having amino acid sequences altered by mutagenesis.

Experimental Procedures

Chimeric molecules were constructed using standard recombinant DNA techniques. Restriction endonucleases and T4 DNA polymerase were from Bethesda Research Laboratories (Gaithesburg, MD). Briefly, appropriate restriction fragments were purified by electrophoresis through GTG agarose gels (FMC, Rockland, ME). After electroelution and ethanol precipitation, these fragments were ligated and the reaction products were transformed into *Escherichia coli* host strain JM101. Transformants were analyzed by restriction digestion.

Site-directed mutants were constructed using a phagemid, pSELECT (Promega, Madison, WI), or a derivative, pS(-), which allows rescue of the (+)-strand.[2] Channel-encoding sequences were subcloned, and single-stranded DNA was rescued by superinfection with M13K07 helper phage. Mutagenic oligonucleotides were annealed to the single-strand DNA in the presence of an oligonucleotide that corrects a frameshift mutation in the β-lactamase gene of the phagemid. Complementary DNA was

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[2] J.P. Adelman, unpublished observation.

synthesized using T4 DNA polymerase and ligase (BRL). The reaction products were transformed in BMH 71-18 *mutS* and grown in liquid culture containing ampicillin; miniprep DNA was isolated and used tc transform JM101. Individual colonies were isolated, single-stranded DNA was rescued, and the nucleotide sequences of the mutagenized regions were determined. Oligonucleotides were synthesized with an Applied Biosystems PCR mate, and DNA sequencing was performed by dideoxy chain termination, according to published methods (Bond et al., 1989). RNA was synthesized *in vitro* from appropriate templates using our published methods (Christie et al., 1989).

Xenopus laevis were housed and oocytes were prepared and injected as previously described (Christie et al., 1989). Currents were recorded using a two-electrode voltage-clamp 24 - 96 hours following injection. Cells were constantly perfused in a solution containing 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM Hepes, pH 7.6, with 0.1 % bovine serum albumin. Cells were clamped at -80 mV, and potassium currents were recorded during 250-msec depolarizing commands to 0 mV every 20 sec. In most experiments, the DTX concentration was increased cumulatively (see Fig. 3). Concentration of DTX giving 50% inhibition (EC_{50}) were estimated by fitting a logistic function

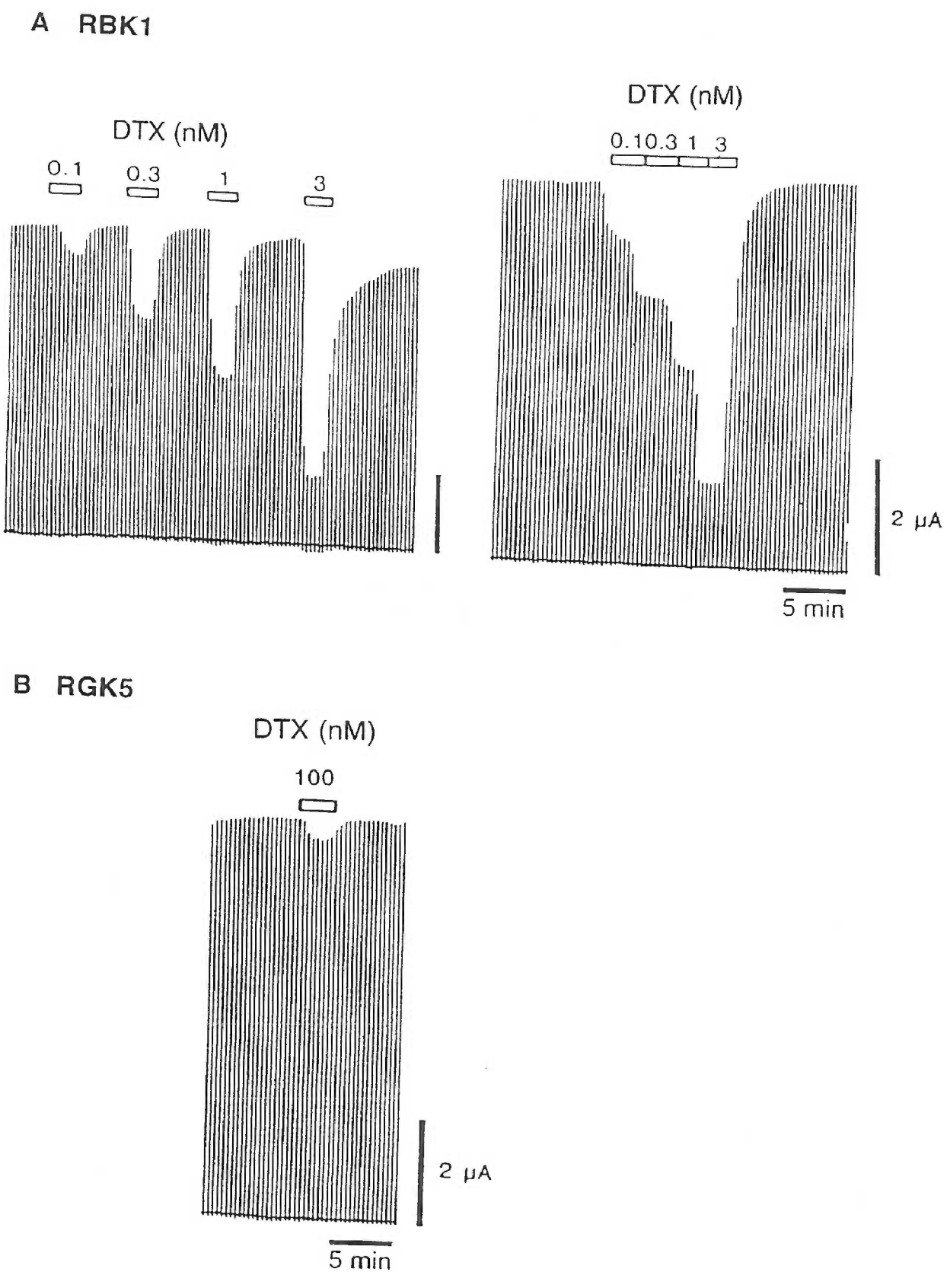
$$I(\%) = 100 \cdot \frac{[DTX]^n}{[DTX]^n + EC_{50}{}^n} \qquad (12)$$

where I was the mean percentage of inhibition observed in three to six oocytes. The Hill coefficient *n was* not significantly different from 1. Three batches of DTX (two from Sigma; one the gift of Dr. J. O. Dolly) were used in these studies; they were kindly purified by the late Dr. R. Hartshorne using gel filtration and ion exchange high performance liquid chromatography. The EC_{50} for inhibition of current through RBK1 channels ranged from 0.3 to 1.5 nM among different batches, but the quantitative comparisons reported are from experiments using the same batch.

Results

We have previously reported the functional characterization of three cloned rat K^+ channels in *Xenopus* oocytes, RBK1 (Christie et al., 1989) (also called RCK1, Stühmer et al., 1988), RBK2 (Christie et al., 1990) (also called RCK2, McKinnon, 1989) and RGK5 (Douglass et al., 1990) (this differs by only two amino acids from RCK3, Stühmer et al., 1989). These channels display delayed rectifier properties, with the main difference between RGK5 and the other two being its inactivation during depolarizations lasting several seconds. The amino acid sequences of the predicted channel proteins are highly homologous yet they differ markedly with respect to DTX sensitivity. Figure 3.A shows the current through RBK1 channels; it was blocked approximately 50% by 1 nM DTX. The block occurred rapidly, and the current quickly recovered its control value when the DTX application was discontinued. RBK2 channels had a DTX sensitivity similar to that of RBK1. Block of RBK1 by 1 nM DTX showed little or no voltage dependence; the block was not more than

Figure 3.


A RBK1
DTX (nM)
0.1
0.3
1
3
DTX (nM)
0.10.3 1 3
2 μA
5 min
B RGK5
DTX (nM)
100
2 μA
5 min

1.2 times greater at +20 mV than at -20 mV. Similar inhibition of the current was observed whether increasing concentrations of DTX were applied sequentially or cumulatively (Fig. 3.A). In contrast, currents in oocytes injected with RGK5 RNA were virtually insensitive to DTX (Fig. 3.B).

As an initial step to localize residues that contribute to DTX sensitivity, chimeric subunits were constructed. RBK2 and RGK5 were used because they both have a *Pst*I restriction site located 5' to the region encoding the S4 transmembrane domain, dividing the coding region approximately in half. The chimera containing the amino terminus of RBK2 and the carboxyl terminus of RGK5 was expressed; the currents were unaffected by 10 nM DTX. In contrast, the channel composed of the amino terminus of RGK5 and the carboxyl terminus of RBK2 was about as sensitive to DTX (EC_{50}, 0.21 ± 0.15 nM; n = 3) as the wild-type RBK2 (EC_{50}, 0.38 ± 0.02 nM; n = 3).

The rapid onset and offset of the action of DTX indicates an extracellular site of action. The carboxyl-terminal portion of the chimeric channel protein is thought to be intracellular except for a 40-residue sequence between membrane-spanning regions S5 and S6 (Guy and Conti, 1990). A well conserved cluster of negative charges is present in the first 10 residues of this region in all homologous cloned potassium channel subunits. Thus, through-space electrostatic interactions between charged residues in RBK1 to DTX were examined by measuring the effectiveness of DTX to inhibit current through RBK1 channels in solutions of different ionic strength. In one series of experiments, the EC_{50} for DTX was 1.5 ± 0.06 nM (n = 3) in normal solution (96 mM NaCl) and 0.36 ± 0.03 nM

(n = 5) in an iso-osmotic solution containing only 48 mM NaCl (sucrose substitution). This 3.5-fold increase in sensitivity to DTX suggests that through-space electrostatic forces play a role in DTX binding, similar to that observed for charybdotoxin (CTX) binding to *Shaker* potassium channels (MacKinnon and Miller, 1989).

Although many of the negatively charged residues in the region between S5 and S6 are conserved among the various cloned K+ channel subunits, there is a correlation between DTX sensitivity and the presence of a negatively charged residue in the position corresponding to Glu^{353} in RBK1 (Table 1, see also Stühmer et al., 1989); this position is occupied by neutral residues in DTX-insensitive subunits such as RGK5. Site-directed mutants were constructed by replacing the targeted amino acids within each parental backbone. Replacement of RBK1 Glu^{353} with Ser {RBK1(E353S)] resulted in a channel 30-fold less sensitive to DTX than wild-type (Fig. 4.A). Many of the cloned subunits that are insensitive to DTX contain a Pro immediately amino-terminal to the position corresponding to Glu^{353} in RBK1; this residue was also changed. Replacement of Ala^{352} with Pro [RBK1(A352P)] produced a 2.5-fold reduction in DTX sensitivity (Fig. 4.A; Table 1). The effects of the two mutations were multiplicative; the double-mutant RBK1(A352P, E353S) was 70-fold less sensitive to DTX than native RBK1 (Fig. 4.A; Table 1). The action of DTX on the mutants lacking the negative charge, although weaker than for RBK1, was still sensitive to ionic strength. RBK1(E353S) was tested with 25 nM DTX, and the inhibition increased from 37.7 ± 1.5 % to 71.0 ± 2.2% (n = 3) when ionic strength was halved; for RBK1(A352P, E353S), 60 nM

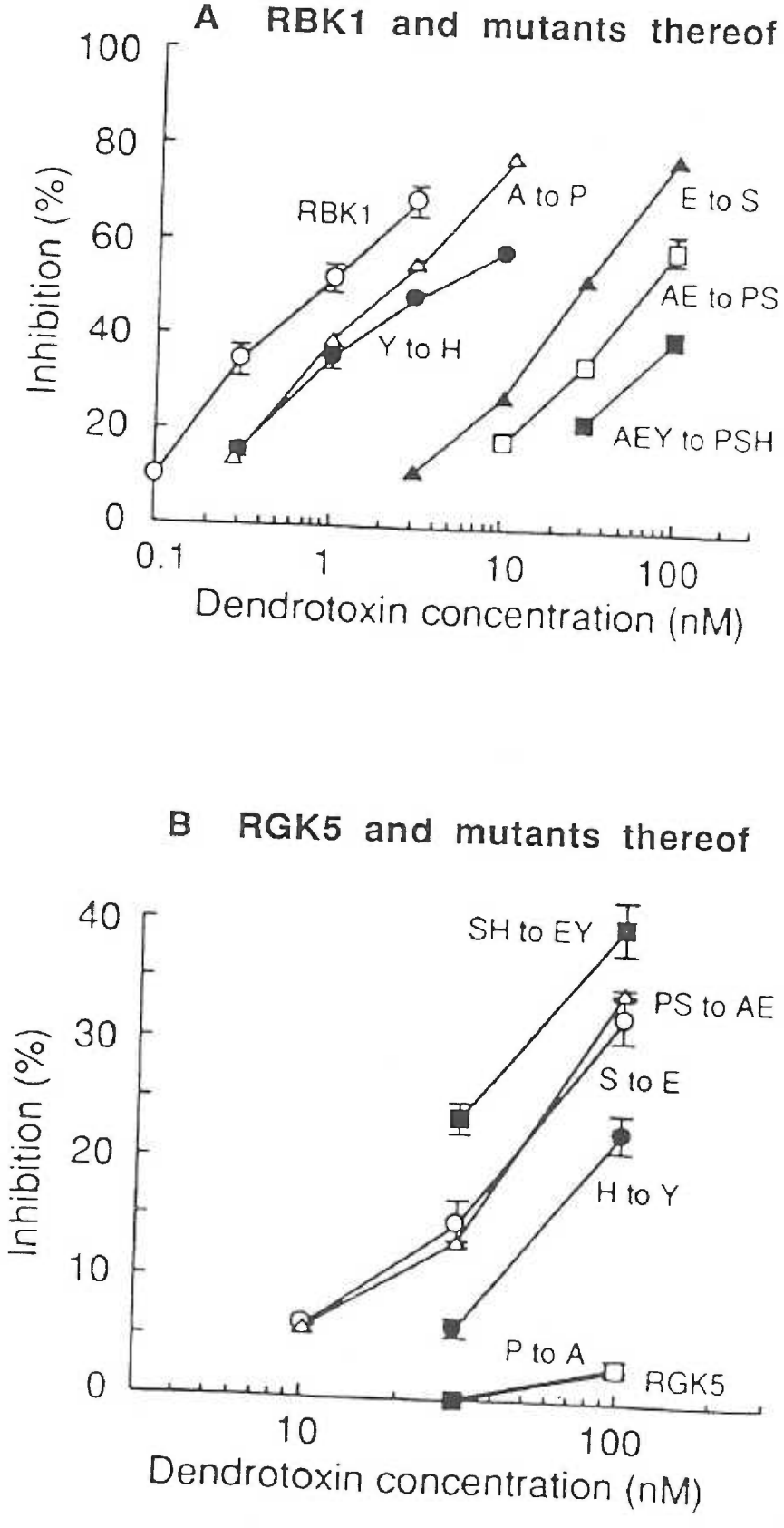
TABLE 1.

S5-S6 region of RBK1 (beginning at F^{346}), RBK2 (beginning at F^{348}) and RGK5 (beginning at F^{368}).

Regions denoted as SS1 and SS2 by Guy and Conti (1990) are indicated beneath the sequences. Number to the right (mean ± s.e.m. of three to six oocytes) are DTX EC_{50} or the inhibition produced by 100 nM DTX.

	EC_{50}	Inhibition by 100 nM DTX
RBK1	nM	%
FAEAEEAESHFSSIPDAFWWAVVSMTTVGYGDMYPVTIGGK	0.96±0.04	
......P.....	2.4 ±0.3	
......PS.....	69 ±11	
.......S.....	26 ±0.2	
.....H.....	2.5 ±0.1	
......PS.....H.....		41 ±2.0
RBK2		
FAEADERDSQFPSIPDAFWWAVVSMTTVGYGDMVPTTIGGK	0.38±0.02	
RGK5		
FAEADDPSSGFNSIPDAFWWAVVTMTTVGYGDMHPVTIGGK		3.1±0.2
......A.....		2.8±0.8
.......E.....		35 ±1.1
......AE.....		32 ±1.9
.....Y.....		23 ±1.6
.......E.....Y.....		40 ±2.2
| SS1 || SS2|		

Figure 4.


A RBK1 and mutants thereof
100
80
60
40
20
0
Inhibition (%)
RBK1
A to P
E to S
AE to PS
Y to H
AEY to PSH
0.1
1
10
100
Dendrotoxin concentration (nM)
B RGK5 and mutants thereof
40
30
20
10
0
Inhibition (%)
SH to EY
PS to AE
S to E
H to Y
P to A
RGK5
10
100
Dendrotoxin concentration (nM)

DTX was used and the respective values were 43.9 ± 3.4 % and 76.2 ± 1.4% (n = 3).

Converse mutants were next constructed in the DTX-insensitive channel RGK5; because the sensitivity is so low, the results are expressed as inhibition caused by 100 nM DTX, rather than EC_{50}. RGK5(S375E) was inhibited 10-fold more than native RGK5 at 100 nM toxin. Changing Pro^{374} to Ala [RGK5(P374A)] affected sensitivity very little, and the double mutant RGK5(P374A, S375E) was no more sensitive to DTX than the single-mutant RGK5 (S375E) (Fig. 4.B, Table 1). Thus, the addition of a negative charge to RGK5 increases DTX sensitivity, but the effect is not as great as the decrease in sensitivity produced by removal of the negative charge from RBK1.

A Tyr in the SS2 region of the S5/S6 loop of voltage-gated potassium channels (Tyr^{379} in RBK1) has recently been shown to lie at the external mouth of the channel, affecting single-channel rectification and TEA binding (MacKinnon and Yellen, 1990; Kavanaugh et al., 1991; MacKinnon et al., 1990). Further, computer modeling (Guy and Conti, 1990) has predicted that this residue lies at the external mouth of the channel; therefore, possible involvement of this amino acid in mediating DTX sensitivity was investigated. Substitution of Tyr by its counterpart in RGK5 [RBK1(Y379H)] resulted in a 2.5-fold decrease in DTX potency. When this change was introduced together with the two changes close to S5 described above, the triple mutant was > 100-fold less sensitive to DTX than RBK1 (Fig. 4.A). Conversely, inhibition of the mutant RGK5(H401Y) by 100 nM DTX was increased 7-fold, compared with

wild-type, and this same substitution in concert with the introduction of Glu^{375} increased DTX inhibition 13-fold, relative to wild-type RGK5 (Fig. 4.B).

The mutant channels examined did not obviously differ in voltage dependence for the parent wild-type channels, and the actio of DTX was also not voltage dependent. In the case of RBK1, RBK1(A352P, E353S), and RGK5(P374A, S375E), the inhibition cause by DTX was not significantly different at -20 mV and +20 mV. RBK1 mutants activated more slowly than wild-type RBK1, and RGK5 mutants activated more rapidly than RGK5, but the activation was in all cases intermediate between the values for RBK1 (< 5 ms at 0 mV) (Christie et al., 1989) and RGK5 (about 20 ms at 0 mV) (Douglass et al., 1990).

Discussion

The present study has identified RBK1 Ala^{352}, Glu^{353}, and Tyr^{379}, three residues located in the loop between transmembrane domains S5 and S6, as being capable of influencing DTX block of the channel. Cation-selective channels in general are thought to have a permeation pathway that is preceded by a cloud of negative charge, which contributes to ion selectivity (Hille, 1984). Experimental evidence for this has been provided by chemical modification studies in which carboxyl-modifying agents have been applied to Na^+ channels or Ca^{2+}-activated voltage-sensitive K^+ channels, resulting in altered ion permeation and gating; such treatments also inhibit binding of saxitoxin and tetrodotoxin to some Na^+ channels and CTX binding to some K^+ channels (Spaulding, 1980; Reed and Raftery,

1976; Worley et al., 1986; MacKinnon and Miller, 1989b). Site-directed mutagenesis allows the carboxyl residues that are important in selectivity and toxin binding to be identified more specifically.

MacKinnon and Miller (1989a) found that substitution of Glu^{422} by Gln reduced the inhibition of current through *Sh*B channels by CTX 3.5-fold, and substitution by Lys reduced the inhibition 12-fold. In the present study, replacement of Glu^{353} by Ser caused about a 30-fold reduction in sensitivity of RBK1 to DTX. Although not in exactly analogous positions by sequence alignment, these two residues are in close proximity in the region between S5 and S6. Thus, it is likely that, despite differences in primary sequence and structure, these two toxins bind to a very similar region of voltage-dependent K^+ channels. This is consistent with the observation that CTX can inhibit ^{125}I-DTX binding to rat brain synaptosomal membranes (Harvey et al., 1989).

Based upon computer modeling of K^+ channel subunit structure, Guy and Conti (1990) have presented a model in which the extracellular loop between transmembrane regions S5 and S6 (about 40 amino acid residues) contains two subsections, termed SS1 and SS2 (see Table 1). These are thought to dip back into the membrane, contributing to the extracellular mouth of the channel and the permeation pathway (Guy and Conti, 1990). Mammalian K^+ channels thus far cloned and tested fall into two classes, sensitive (EC_{50} < 10 nM) or insensitive (EC_{50} > 200 nM) to DTX (Christie, et al., 1989; Stühmer et al., 1989; Swanson et al., 1990, Stühmer et al., 1988). A Tyr residue in SS2 (Tyr^{379} in RBK1) has been identified as a major

determinant of the binding of TEA (MacKinnon and Yellen, 1990; Kavanaugh et al., 1991). The present results indicate that it also participates in DTX binding. Replacement of this Tyr in RBK1 with its counterpart (His) in RGK5 reduced the affinity for DTX by a facto of about 2.5. The effect appeared to be relatively independent of the substitutions above in the SS1 region (Ala^{352} and Glu^{353}), and the converse substitutions in RGK5 had the expected effects (Table 1).

The mutations made in this study resulted in the expression of channels that had altered sensitivities for DTX of up to 150-fold, a change in binding energy equivalent to about 3 kcal/mol. Although this contribution is very significant, other residues must also play a role, because the changes in apparent K_D observed for single amino acid substitutions never amounted quantitatively to the difference between RGK5 and RBK1. The RBK1 mutant with all three substitutions [RBK1(A352P, E353S, Y379H)] had about the same sensitivity to DTX as the most sensitive RGK5 mutant tested [RGK5(S375E, H381Y)]. Some of the other, as yet unidentified, residues that contribute to DTX binding likely participate in ionic interactions, because the blocking potency of DTX was still increased by lowering the ionic strength in the Glu→Ser mutant. Given the size of the DTX molecule and the high affinity with which it binds, it seems likely that the bound complex is stabilized by a number of interactions, unlike the case with the structurally much simpler molecule TEA (MacKinnon and Yellen, 1990; Kavanaugh et al., 1991).

Although first thought to be a presynaptic toxin, because of its action to increase transmitter release (Harvey and Anderson, 1985;

Harvey and Karlsson, 1980; Barrett and Harvey, 1979), DTX is now known to block non-inactivating, voltage-dependent K^+ currents in the membrane of neuronal somata such as dorsal root ganglion cells and hippocampal pyramidal neurons (Stansfield et al., 1986, Moczydlowski et al., 1988; Halliwell et al., 1986; Penner et al., 1986). Action potential broadening in presynaptic fibers is, therefore, thought to account for the enhanced transmitter release. The effective concentrations in guinea pig dorsal root ganglion cells are the same as those that block RBK1 and RBK2 in *Xenopus* oocytes. The present finding that DTX sensitivity can be attributed in large measure to the presence of discrete amino acid residues may eventually help determine the molecular species of channels that are expressed in presynaptic nerve terminals.

Acknowledgments

We thank Dr. Oliver Dolly for a gift of DTX used in some early experiments, the late Dr. Robert Hartshorne for the purification of DTX, Yan-Na Wu for oocyte injections, and Chris Bond and Dr. Jim Douglass for useful discussions.

CHAPTER 2

COOPERATIVE INTERACTIONS AMONG SUBUNITS OF A VOLTAGE-DEPENDENT POTASSIUM CHANNEL: EVIDENCE FROM EXPRESSION OF CONCATENATED cDNA

R. S. Hurst, M. P. Kavanaugh, J. Yakel, J. P. Adelman, and R. A. North

Summary

Four copies of the coding sequence for a voltage-dependent potassium channel (RBK1, rat Kv1.1) were ligated contiguously and transcribed *in vitro.* The resulting RNA encodes four covalently linked subunit domains, called [4]RBK1. Injection of this RNA into *Xenopus* oocytes resulted in the expression of voltage-dependent potassium currents. A single amino acid substitution, Tyr→Val, located within the outer mouth of the pore, introduced into the equivalent position of any of the four domains, reduced affinity for external tetraethylammonium by approximately the same amount. In constructs containing 0, 1, 2, 3, or 4 Tyr residues, the free energy of binding tetraethylammonium was linearly related to the number of Tyr residues. A different amino acid substitution, Leu→Ile, located in the S4 region, was made in the equivalent position of one, two, three or four domains. The depolarization required for channel activation increased approximately linearly with the number of Ile residues, whereas models of independent gating of each domain predict a marked non-linearity. Expression of this concatenated channel provides direct evidence that voltage-dependent potassium

channels have four subunits positioned symmetrically around a central permeation pathway and that these subunits interact cooperatively during channel activation.

Introduction

Subunits of voltage-dependent K^+ channels are thought to have six α-helical membrane-spanning domains (S1-S6) and two shorter β-sheeted segments that contribute to the ion conducting pore (termed P, or SS1/SS2; see Guy and Conti, 1990; Durell and Guy, 1992). There is evidence that the channel is made by the association of four such subunits, analogous to voltage-dependent Na^+ and Ca^{2+} channels (MacKinnon, 1991; Catterall, 1988; Jan and Jan, 1989). Diversity can be generated by heteropolymerization of different subunits expressed in the same cell (Beck and Pongs, 1990; Drew et al., 1992; for review, see Salkoff et al., 1992), and heteropolymeric channels have been identified after injecting RNAs encoding two subunits into *Xenopus* oocytes (Christie et al., 1990; Isacoff et al., 1990; Ruppersberg et al., 1990). Heteropolymerization occurs among channel subunits within the *Shaker*, *Shab*, *Shaw*, and *Shal* families of channels, although not between subunits belonging to different families (Salkoff et al., 1992; Covarrubias, 1991). The injection of two RNAs in various proportions has been used to express *mixtures* of channels in proportions expected from binomial theory (MacKinnon, 1991; Kavanaugh et al., 1992), but that approach relies on untested assumptions of equal translation and random association of subunits. More importantly, such co-injection experiments do not

allow one to vary systematically the subunit composition of a *singl* homogeneous channel species.

Tetrameric channels of known subunit composition are necessary to test models of subunit interaction. For example, the Hodgkin and Huxley (1952b) models of the action potential implied that four subunits (or gating particles) operate independently during activation of the K^+ current by depolarization. However, gating currents of the *Shaker* H4 channel measured using the cut-open oocyte technique suggest otherwise (Bezanilla et al., 1991). The time course of ON gating currents indicates that during activation, transitions that occur later (*i.e.* closer to the open state) are faster than those which occur earlier. This suggests that the conformational change that a subunit undergoes when it is activated becomes progressively easier as surrounding subunits are activated (see also Papazian et al., 1991).

The main purpose of the present experiments was to express K^+ channels of known subunit composition so as to test the effects of mutations in individual subunits on channel activation. Rather than using separate subunits, the potassium channel was expressed as a contiguous polypeptide of four concatenated cDNAs. A rat homolog of *Shaker* was used (RBK1, Ref. Christie et al., 1989; also called Kv1.1, Ref. Chandy, 1991), and the concatenated cDNA and channel are termed [4]RBK1. The first part of the paper describes experiments which verify that the resultant channel was indeed formed as a continuous polypeptide with four domains. To do this, we studied the effects of a single amino acid mutation in one or more subunits on the action of extracellular TEA, which is known to

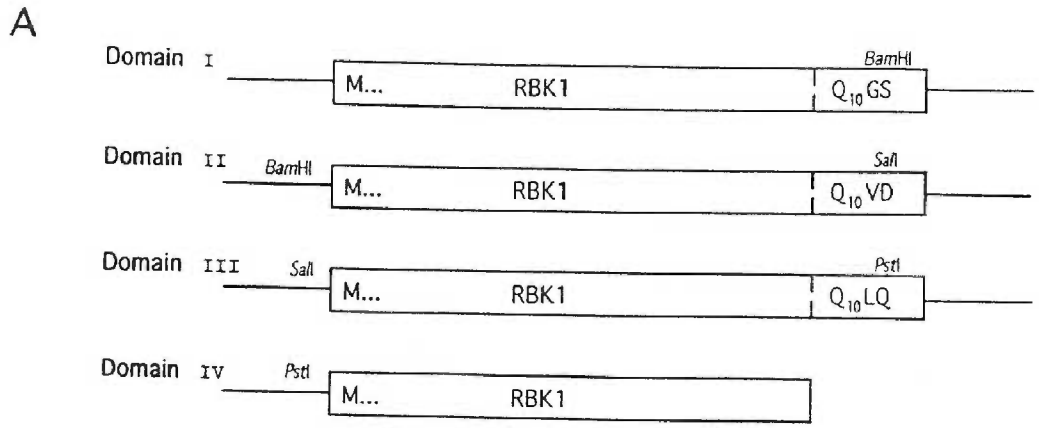
block the channel by entering the conduction pore (Kavanaugh et al., 1991; MacKinnon and Yellen 1990; Heginbotham and MacKinnon, 1992). These experiments also provide evidence that the external TEA binding site is made from approximately equal contributions by each of the four Tyr residues.

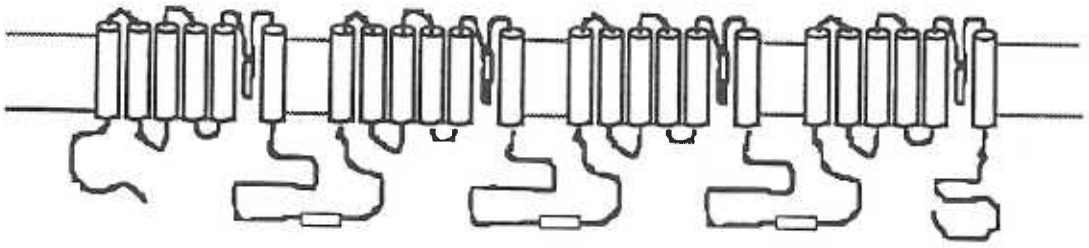
The second part of the paper describes experiments to determine whether there is cooperative interaction among subunits during the voltage-dependent activation (opening) of the channel. The voltage dependence of activation is markedly affected by a single-point mutation of a hydrophobic residue within the S4 region of a *Shaker* subunit (Lopez et al., 1991; McCormack, 1990); in that case, the expressed channels would have four subunits each bearing the mutation. Therefore, a similar mutation was introduced into the S4 region of one, two, three, or four domains of the concatenated channel, and the voltage dependence of their activation was compared with that expected for independent non-interacting domains.

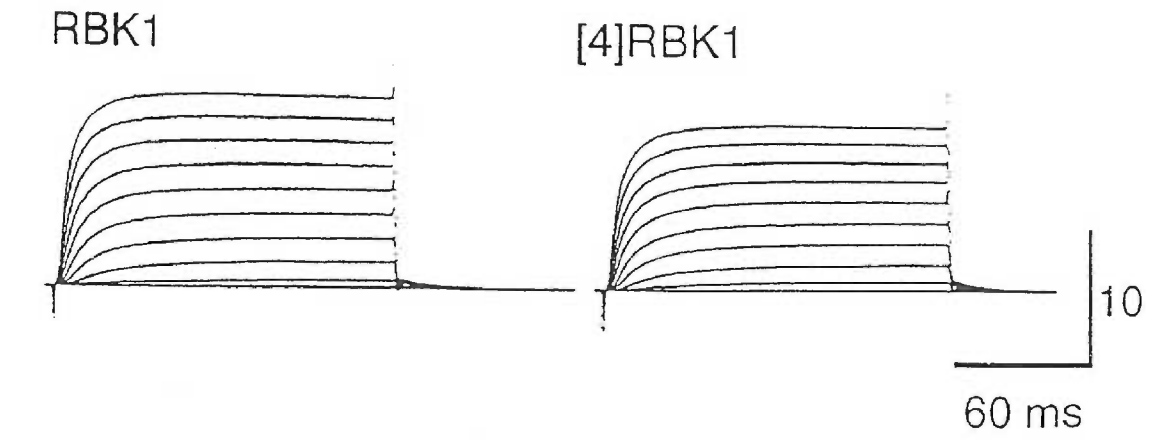
Experimental Procedures

Concatenated cDNA Construction and site-directed Mutagenesis. RBK1 coding sequences were concatenated by making four unique cDNAs of RBK1 that could be ligated together in a predetermined orientation (Fig. 5.A). The amino-terminal subunit (domain I) was constructed by introducing 36 bases of DNA, encoding 10 Gln residues, at the 3' end of an RBK1 cDNA. The Gln linker was engineered such that the first Gln codon replaced the natural RBK1 stop codon and that the correct reading frame was

Figure 5.


A
Domain I
M...
RBK1
BamHI
Q_{10}GS
Domain II
BamHI
M...
RBK1
SalI
Q_{10}VD
Domain III
SalI
M...
RBK1
PstI
Q_{10}LQ
Domain IV
PstI
M...
RBK1


B


C
RBK1
[4]RBK1
10
60 ms

maintained. The Gln linker region contained a *Bam*HI restriction sit immediately 3' to the 10th Gln codon which added two amino acids (Gly and Ser). The second subunit (domain II) was constructed by introducing a *Bam*HI restriction site immediately 5' to the usual RBK1 translation initiation codon (ATG). In addition, a linker region of 10 Gln followed by a *Sal*I restriction site (encoding Val and Ser) was introduced at the 3' end, replacing the usual stop codon in RBK1 The third subunit (domain III) was constructed in an analogous manner by inserting a *Sal*I restriction site 5' to the initiator ATG and a linker region of 10 Gln followed by a *Pst*I restriction site (encoding Leu and Gln) at the 3' end. The fourth subunit (domain IV) was generated by inserting a *Pst*I site 5' to the initiating ATG. Domain I was ligated to domain II across the *Bam*HI site, and domain III was ligated to domain IV across the *Pst*I site to generate two unique RBK1 doublets. Ligation products were transformed into *Escherichia coli* host strain JM109, and individual clones were analyzed by restriction analysis and DNA sequencing. Appropriate restriction fragments were isolated and ligated across the *Sal*I restriction site to generate the [4]RBK1 construct. This ligation reaction was transformed into *Escherichia coli* host strain SURE (Stratagene, La Jolla, CA). The resulting plasmid was verified to contain four RBK1 coding sequences joined in the same reading frame with the Gln-encoding linker regions by restriction analysis and DNA sequencing.

RBK1 subunits containing the mutations $Tyr^{379} \rightarrow Val$ or $Leu^{305} \rightarrow Ile$ were generated by site-directed mutagenesis (Hurst et al., 1991), using the individual subunit coding sequences which

already contained the Gln linker regions and the restriction sites. The DNA constructs which encoded the mutated subunits were ligated to other RBK1 wild type or mutated cassettes, as described above, to generate the various [4]RBK1 constructs. All plasmid constructs were made in the pS- vector, a derivative of pSELECT (Promega, Madison, WI) in which the f1 origin of replication had been reversed to permit rescue of the coding strand by helper phage-mediated superinfection.

Electrophysiology. Oocytes (stages V-VI) were injected with approximately 1 ng of *in vitro* transcribed RNA. Membrane currents were recorded 24-96 hours later by a two-electrode voltage clamp (Christie et al., 1989). The voltage dependence of activation was measured by clamping the oocyte membrane at -80 mV and depolarizing to a series of test potentials. Leak-subtracted peak currents were divided by the driving force (assuming a reversal potential of -90 mV) to obtain the conductance, and this was expressed as a fraction of its maximal value (G/G_{max}). Inhibition by TEA was measured by clamping the oocytes at -80 mV and recording currents during 250-ms pulses to 0 mV, repeated every 30 s. TEA was applied by superfusion, and the concentration was increased cumulatively. Current amplitude was measured when it reached its new steady state in the presence of each concentration of TEA.

Data analysis. Inhibition by TEA (I, percent) of singly injected oocytes was fitted by sum of squares minimization to I(%) equals

$$100 \cdot \frac{[TEA]}{[TEA] + K} \qquad (13)$$

to provide an estimate of K, the apparent dissociation constant. When mixtures of two channel species were expressed (*e.g.* [4]RBK1 + RBK2), the dose-response curves were fitted to I(%) equals

$$\left((100\cdot r)\cdot\frac{[\mathrm{TEA}]}{[\mathrm{TEA}]+K_1}\right)+\left(\left(100\cdot(1-r)\right)\cdot\frac{[\mathrm{TEA}]}{[\mathrm{TEA}]+K_2}\right) \qquad (14)$$

or

$$\left((100\cdot r_1)\cdot\frac{[\mathrm{TEA}]}{[\mathrm{TEA}]+K_1}\right)+\left((100\cdot r_2)\cdot\frac{[\mathrm{TEA}]}{[\mathrm{TEA}]+K_2}\right)+\left(100\cdot(1-r_1-r_2)\cdot\frac{[\mathrm{TEA}]}{[\mathrm{TEA}]+K_3}\right) \qquad (15)$$

were K_1 and K_2 are the apparent TEA equilibrium constants for the channels when expressed singly, and K_3 is the apparent dissociation constant for *all* heteropolymeric channels. The mean inhibition (n = three to eight oocytes) was used for this fitting. For estimation of the apparent affinity K of TEA for individual channel species, the results from single oocytes (four to eight TEA concentrations) were fitted to logistic functions (see above). K was converted to the change in free energy (ΔG) that occurs upon dissociation of TEA according to

$$\Delta G = -RT\ln K \qquad (16)$$

The normalized conductance (G/G_{max}) was fit to

$$G / G_{max} = \frac{1}{1 + exp[(V_{0.5} - V) / k]} \quad (17)$$

were $V_{0.5}$ is the potential at which $G/G_{max} = 0.5$, V is the depolarizing potential, and k is the number of mV required to cause e-fold shift in conductance. For the constructs containing Leu→Ile mutations, the activation curves predicted for independently activating domains (subunits) were obtained from

$$G / G_{max} = \left(\frac{1}{1 + exp[(V_L - V) / k_L]}\right)^n \cdot \left(\frac{1}{1 + exp[(V_I - V) / k_I]}\right)^{(4-n)} \quad (18)$$

where n is the number of domains containing Leu; (4-n) domains containing Ile. V_L, V_I, k_L, and k_I are the values of $V_{0.5}$ and k for the individual domains containing Leu or Ile; these were estimated by least squares fitting to the data for n=4 and n=0, respectively.

Results

Expression of [4]RBK1. Currents in oocytes injected with the [4]RBK1 RNA activated within a few milliseconds (τ at 0 mV was 3.6 ± 0.6 ms, n = 5) (Fig. 5.C). The voltage dependence of activation was well fit by a Boltzmann function, where $V_{0.5}$ was -13.5 ± 6.4 mV and k was 13.0 ± 0.9 mV (n = 5). The values for RBK1 in parallel experiments were τ = 5.4 ± 0.7 ms, $V_{0.5}$ -16.9 ± 2.1 mV, k = 11.1 ± 1.1 mV n = 5.) The reversal potential of the current changed with external K^+ concentration as expected from the Nernst equation for a K^+ selective pore.

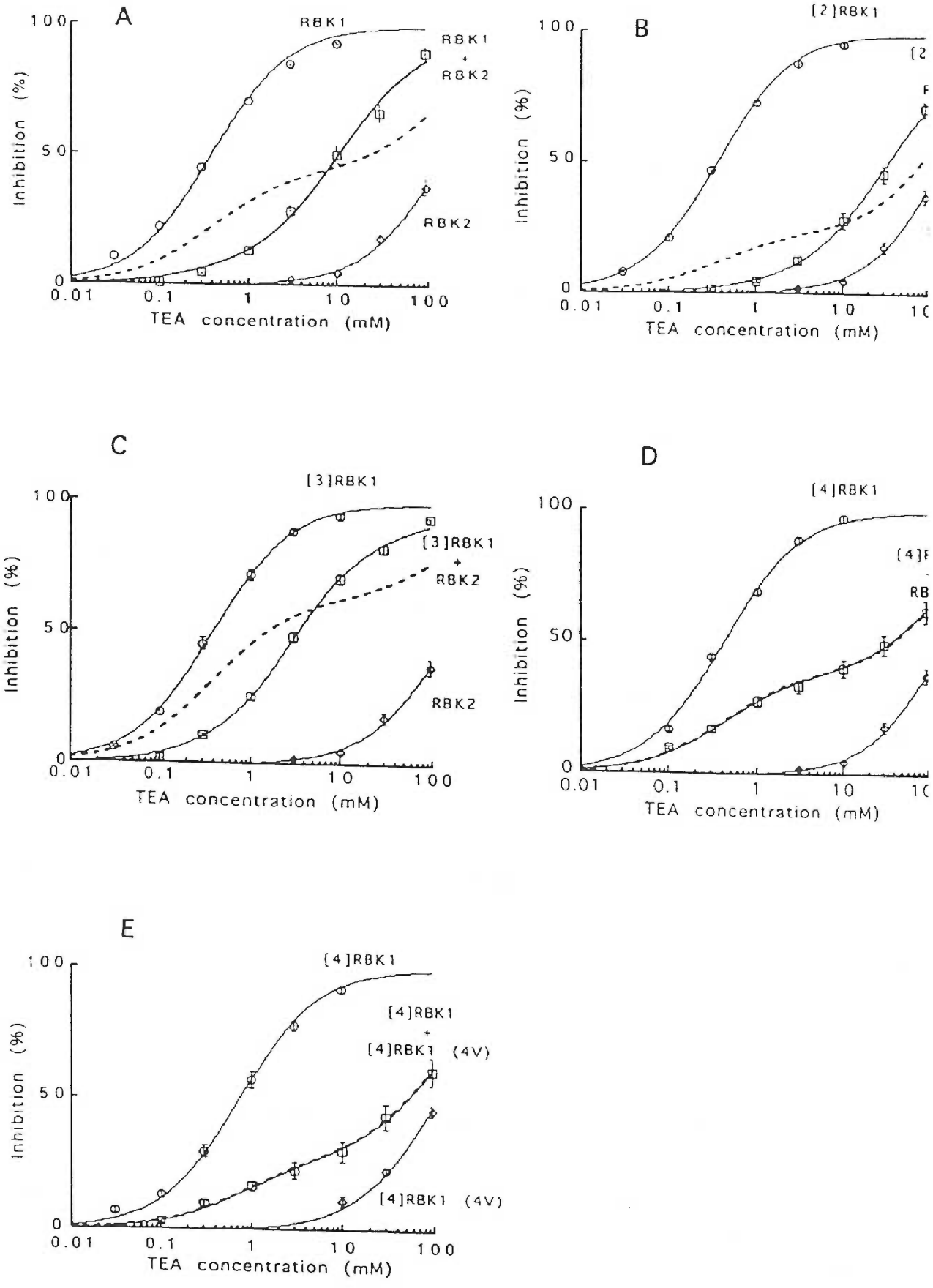
The concentration of TEA that gave half-maximal current inhibition (apparent equilibrium constant, K) was similar to that fo RBK1 (Fig. 6), and the inhibition was well described by

$$I(\%) = 100 \cdot \left(\frac{[TEA]^n}{[TEA]^n + K^n} \right) \qquad (19)$$

where the averaged inhibition data were fitted by a Hill coefficient n of 0.95. The inhibition of [4]RBK1 current by α-dendrotoxin (K = 0.5 ± 0.2 nM, n = 3) was also similar to that observed with RBK1 (K = 0.3 ± 0.1 nM, n = 3).

[4]RBK1 forms channels as a single intact polypeptide. It is possible that [4]RBK1 formed channels by polymerization of individual subunits that resulted from either RNA degradation in the oocyte, translation initiation at internal methionines, or proteolysis. They could also form by polymerization of intact [4]RBK1 molecules. In any of these cases, polymerization should be possible with other distinct K^+ channel subunits if these are co-expressed in the same oocyte. RBK2 is a K^+ channel subunit that is homologous to RBK1 but is much less sensitive to TEA; it readily heteropolymerizes with RBK1 (Christie et al., 1990). We therefore co-expressed [4]RBK1 with RBK2. The concentration-response curves for inhibition of current by TEA were well fit by the sum of two logistic functions (broken curve in Fig. 6.D; from equation 14), demonstrating that current passes only through two distinct channel populations, [4]RBK1 and homomeric RBK2 channels. In this case, there was no improvement in the fit by the addition of a third

Figure 6.


A
RBK1
RBK1 + RBK2
RBK2
Inhibition (%)
100
50
0
0.01
0.1
1
10
100
TEA concentration (mM)
B
[2]RBK1
Inhibition (%)
TEA concentration (mM)
C
[3]RBK1
[3]RBK1 + RBK2
RBK2
Inhibition (%)
TEA concentration (mM)
D
[4]RBK1
Inhibition (%)
TEA concentration (mM)
E
[4]RBK1
[4]RBK1 + [4]RBK1 (4V)
[4]RBK1 (4V)
Inhibition (%)
TEA concentration (mM)

logistic function (solid curve in Fig. 6.D; from equation 15), which would account for any population of channels with a TEA sensitivity intermediate to [4]RBK1 and RBK2. This result is in contrast to experiments with the co-expression of RBK1 and RBK2. As reported previously (Christie et al., 1990; Kavanaugh et al., 1992), the TEA concentration-response curve clearly indicated the formation of channel species having intermediate sensitivity to TEA (Fig. 6.A). In this case, addition of a third logistic function (solid curve) provided a significantly better fit to the data as compared to the simple sum of two logistic functions (broken curve). Similarly, concatenated channel constructs having either two ([2]RBK1, Fig. 6.B) or three ([3]RBK1, Fig. 6.C) linked RBK1 domains were able to incorporate free RBK2 subunits as evidenced by the improvement in fit by adding a third logistic function (solid curve). Finally, we have previously demonstrated that Tyr→Val substitution at position 379 in RBK1 results in the expression of a current that is approximately 30-fold less sensitive to TEA; co-expression of [4]RBK1 with [4]RBK1 containing the Tyr→Val substitution in each domain provided no evidence for heteropolymerization (Fig. 6.E). This suggests that [4]RBK1 molecules did not readily polymerize, instead channels were formed from individual molecules.

The expression of two contiguous potassium channel subunits has been used previously to provide evidence for heteropolymerization (Isacoff et al., 1990). When only one of the domains contained the Tyr→Val substitution, the resulting currents had a sensitivity to TEA that was intermediate between that found when both domains contained Tyr and when both domains contained

Val (Kavanaugh et al., 1992; Heginbotham and MacKinnon, 1992). In other experiments (Kavanaugh et al., 1992), mixtures of single subunits were expressed which contained either Tyr or Val, and the contribution of the Tyr residue from each subunit was estimated by fitting the TEA inhibition curves. These results indicated that four Tyr residues, one from each subunit, take part in the binding of a single TEA ion (Kavanaugh et al., 1992; Heginbotham and MacKinnon, 1992).

Therefore, concatenated channels were made in which the Tyr of the first, second, third and fourth domains was replaced by Val, either singly or in combination. Each domain of [4]RBK1 and its mutants are denoted by a Roman numeral I, II, III, and IV; domains having the Tyr→Val substitution are indicated with a superscript V (^{V}). For example I^{V}-II-III-IV indicates a mutant form of [4]RBK1 in which the Tyr→Val substitution was introduced into the first domain. The inhibition by TEA of the currents in oocytes expressing these channels is shown in Figure 7. The successive introduction of Tyr residues into [4]RBK1 progressively decreased the sensitivity to TEA, and in each case the inhibition was fit by a logistic function with unit Hill coefficient. Substitution of the same number of Tyr residues by Val had approximately the same effect independent of the domains into which they were substituted (Table 2).

Cooperative interaction between subunits during activation. The RBK1 homolog in which Leu^{305} was mutated to Ile (Leu→Ile) was expressed; the currents activated only at very positive membrane potentials. Therefore, RBK1(L305I) was used as the basis

Figure 7.

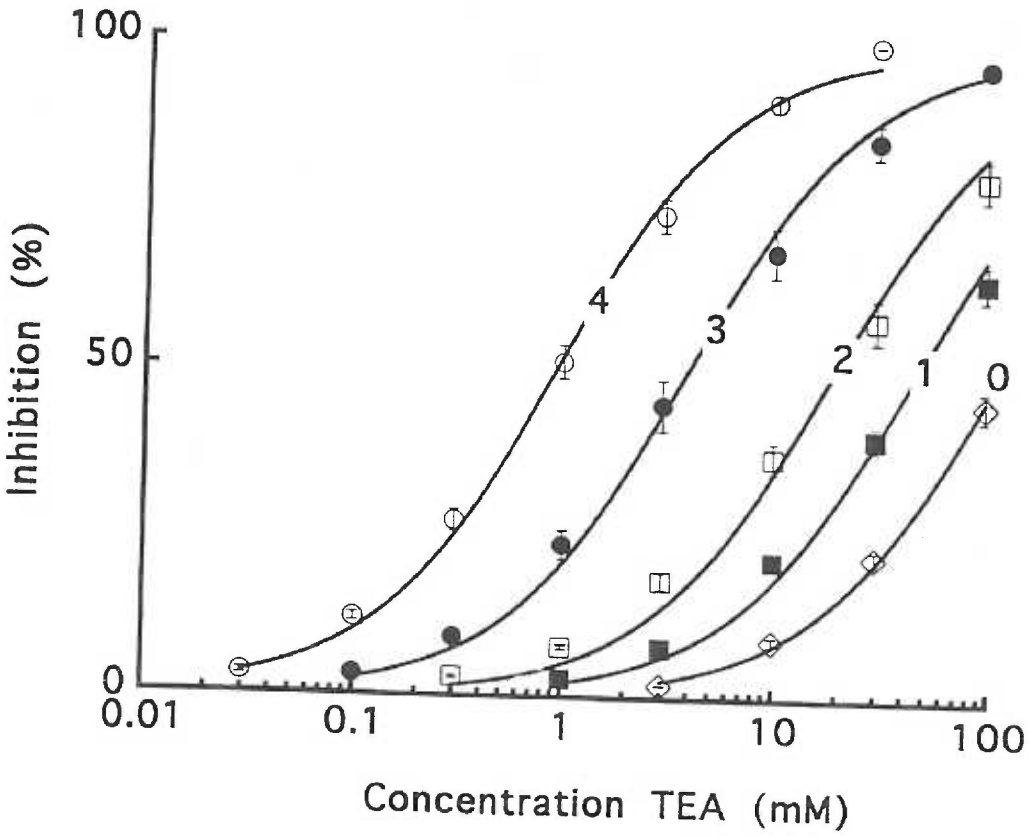

100
50
0
Inhibition (%)
0.01
0.1
1
10
100
Concentration TEA (mM)
4
3
2
1
0

Table 2. Changes in free energy upon dissociation of TEA from [4]RBK1. I-II-III-IV is [4]RBK1, the concatenate of four RBK1 subunits. Superscript V (^{V}) indicates a domain having the Tyr→Val substitution. The Tyr was in the equivalent position in each domain (residue 379 in domain I, 886 in domain II, 1393 in domain III, and 1900 in domain IV). Mean values are independent of the domain that contained Tyr→Val substitution.

Construct	ΔG (kJ/mol)	s.d.	n
I-II-III-IV	17.3	0.8	9
I^{V}-II-III-IV	12.7	0.4	15
I-II^{V}-III-IV	14.0	0.8	7
I-II-III^{V}-IV	13.2	0.8	9
I-II-III-IV^{V}	14.6	1.1	10
Mean	13.5	1.1	41
I^{V}-II^{V}-III-IV	10.2	0.7	16
I^{V}-II-III^{V}-IV	8.8	0.8	18
I-II^{V}-III^{V}-IV	9.3	0.7	15
I-II^{V}-III-IV^{V}	10.4	0.7	14
Mean	9.7	0.9	63
I^{V}-II^{V}-III^{V}-IV	7.0	1.6	20
I^{V}-II^{V}-III-IV^{V}	8.2	1.2	14
I^{V}-II-III^{V}-IV^{V}	7.3	1.3	13
I-II^{V}-III^{V}-IV^{V}	6.9	1.4	13
Mean	7.4	1.4	60
I^{V}-II^{V}-III^{V}-IV^{V}	5.2	1.0	17

to construct and express concatenated channels in which this Leu residue in S4 was changed to Ile in one, two, three, or four domains.

Figure 8. shows that the voltage dependence of activation was shifted progressively to more depolarized potentials as each Leu residue was sequentially replaced by Ile. The degree of shift was approximately equal, ≈ 12 mV/Leu→Ile substitution, one into each domain. Also shown in Figure 8. are the expected curves if each of the four domains activated independently (broken lines from equation 18). Clearly, much less depolarization is required to activate channels having one, two or three Ile-containing domains than expected if each domain activated independently. This implies positive cooperativity among the domains of [4]RBK1 and, presumably, the subunits of native K^+ channels.[3]

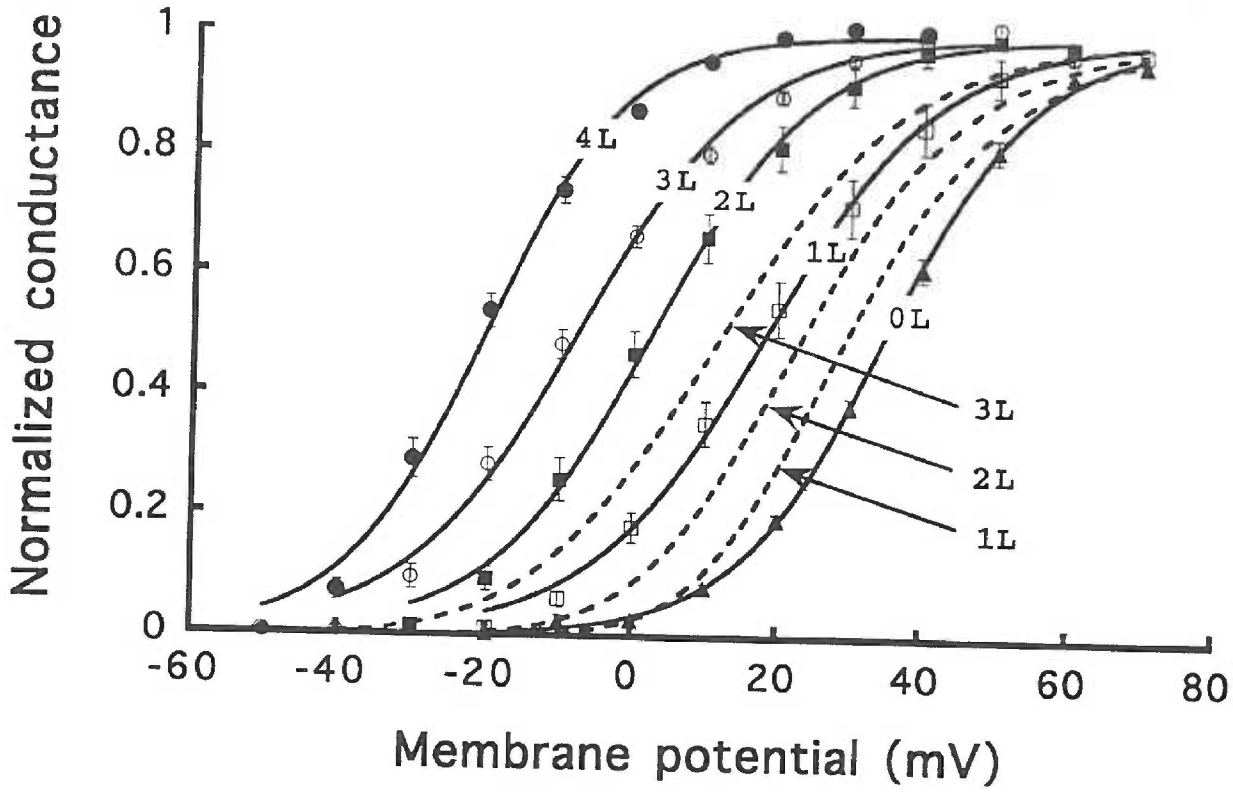
Discussion

The expression of [4]RBK1 resulted in functional K^+ channels with properties that did not differ markedly from those of RBK1. The external vestibule of the channel appears to form normally, as evidenced by the finding that TEA and DTX blocked [4]RBK1 channels at the same concentrations as those required to block the RBK1 channels. The results depicted Figure 6. allow us to conclude that within the resolution of our measurements, [4]RBK1 channels form exclusively as intact polypeptides. [4]RBK1 did not polymerize with

---

[3] The activation curve of RBK1 (L3051) was shifted approximately +50 mV as compared to wild type RBK1 currents. This shift was not different from that observed when the corresponding mutation was introduced into each domain of [4]RBK1 (0L Fig. 8). This suggest that the apparent interaction observed between domains of [4]RBK1 was not a result of linking the channel subunits.

Figure 8.

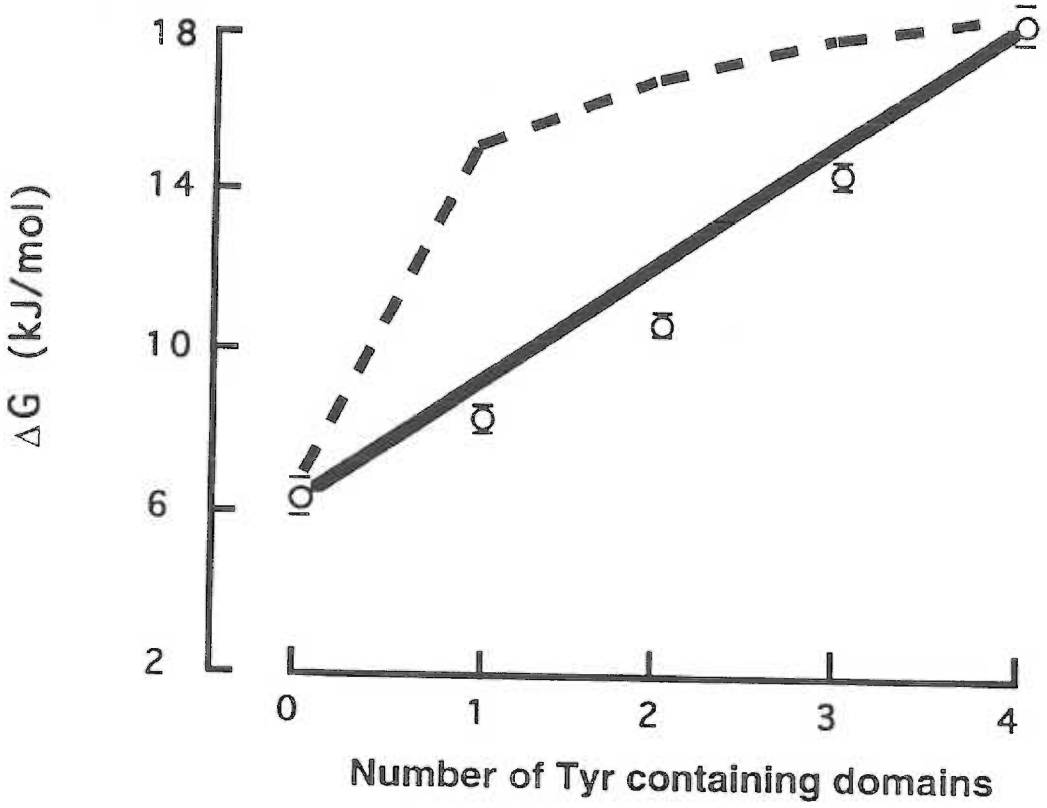

Normalized conductance
1
0.8
0.6
0.4
0.2
0
-60
-40
-20
0
20
40
60
80
Membrane potential (mV)
4L
3L
2L
1L
0L
3L
2L
1L

RBK2 (Fig. 6.D), and [4]RBK1 did not polymerize with the TEA insensitive version of [4]RBK1 (I^V-II^V-III^V-IV^V) (Fig. 6.E). In contrast, monomeric RBK1 subunits readily heteropolymerize both with RBK2 subunits (Fig. 6.A; see also Christie et al., 1990) and with RBK1 (Y379V) subunits (Kavanaugh et al., 1992). Simulations of the dose-response curves for the mixture of [4]RBK1 with RBK2 (Fig. 6.D) indicated that any heteropolymeric channel species of intermediate TEA sensitivity contributed less than 5% to the total current.

Previous experiments using mixtures of wild-type and TEA insensitive mutant channel subunits, and tandem constructs, have indicated that the Tyr^{379} residue on each of the four subunits of the RBK1 channel contributes to the binding of TEA (Kavanaugh et al., 1992; Heginbotham and MacKinnon, 1992). The present results (Table 2) show that [4]RBK1 forms channels with essentially the same symmetry as RBK1 itself, and they provide the most direct evidence to date that each of the four Tyr residues contributes approximately equally to the TEA binding site (Fig. 9.).

The depolarization required to activate concatenated channels with a Leu→Ile substitution in one or more S4 segments was considerably less that expected if the domains operated independently during activation. For example, for the construct having two domains with the Leu→Ile substitution, the model of independent activation indicates that $V_{0.5}$ is 23 mV (broken line arrowed as 2L in Fig. 8). In other words, at 23 mV the expected probability of channel being open (p_o) is 0.5, and the energies of the open and closed states are equal. The experimentally observed value at this potential was 0.87 (from solid line marked as 2L in Fig. 8).

Figure 9.



This increased p_o corresponds to an energy difference of $RT/n[(1-p_o)/p_o]$ or 4.6 kJ/mol; presumably this is supplied by cooperative interactions among the domains.

It seems likely that concatenated channels may provide a useful model system for the kind of quantitative studies of subunit interaction that have been attempted for other tetrameric proteins (*e.g.* Ackers et al., 1992; Giudici-Orticoni et al., 1990). The overall similarities between the properties of this concatenated channel and that formed from individual RBK1 subunits provides confidence that similar interactions would occur among the subunits of multimeric native channels.

Acknowledgment. We thank Yan-Na Wu for expertly injecting oocytes.

CHAPTER 3

EFFECTS OF A PROLINE RESIDUE INTRODUCED INTO ONE OR TWO S4 SEGMENTS OF A CONCATENATED POTASSIUM CHANNEL.

R. S. Hurst, R. A. North, and J. P. Adelman

Summary

Voltage-dependent calcium and sodium channels have a proline residue in the S4 segment of either one or two domains. The functional unit of these channels is a single polypeptide encoding four homologous but not identical domains. Voltage-dependent potassium channels, formed by the association of four subunits, do not have a proline residue in the S4 segment. The present work describes the effect on current activation and deactivation caused by a proline residue introduced into one or two S4 segments of [4]RBK1, a concatenated voltage-dependent potassium channel comprising four linked RBK1 subunits. Two positions within the S4 segment of RBK1 (Ile^{302} and Leu^{305}) were substituted by proline; these positions were chosen based on amino acid alignments with sodium and calcium channel S4 segments. Voltage-dependent potassium currents were evoked from oocytes injected with constructs having one proline containing S4 segment, but functional channels did not appear to form with two proline containing S4 segments. The Ile→Pro substitution in a single S4 segment shifted the activation curve to more depolarized potentials without

changing the slope. The Leu→Pro substitution in a single S4 segment shifted the activation curve to more depolarized potentials and decreased the slope. Possibly, the Leu→Pro substitution interfered with or even prevented the contribution of that S4 segment to channel opening.

Introduction

Voltage-dependent ion channels undergo conformational changes between conducting and non-conducting states. The transition rates between some states are governed by the potential difference across the membrane. The voltage-dependence of channel activation is largely attributed to the S4 segments, the fourth putative membrane spanning region in each domain of Na^+ and Ca^{2+} channels or in each subunit of K^+ channels. S4 segments, predicted to be α-helical in structure, were first postulated to be involved in the voltage dependence of channel activation due to a regular repeat of positively charged amino acids (see Noda et al., 1984). Evidence in support of this hypothesis has been provided by amino acid substitutions for both charged (Auld et al., 1990; Stühmer et al., 1989; Logothesis et al., 1992; Papazian et al., 1991; Liman et al., 1991) and hydrophobic (Lopez et al., 1991; McCormack et al., 1991) residues within this region. Most models of channel activation predict that the S4 segment is displaced across the electric field upon depolarization of the membrane potential (Guy and Conti, 1990; Durell and Guy, 1992; Catterall, 1988). The energy for this reaction is supplied by electrostatic forces between the electric field and the charge and/or dipole moment on the S4 segment. Conformational

changes associated with the voltage-dependent movement of the S4 segments are thought to lead to the opening of the channel pore (Durell and Guy, 1992).

A conserved Pro residue is found in the central region of either one or two S4 segments of voltage-dependent Na^+ and Ca^{2+} channels; Pro residues are not in the S4 segment of any *Shaker* like voltage-dependent K^+ channel. Unlike other residues, Pro forms a ring structure in which the last carbon atom on the side chain is bonded to the main chain nitrogen atom. This prevents the characteristic H-bonding pattern of an α-helix and also provides some steric hindrance, usually resulting in a bend at that position of the helix (Chou and Fasman, 1974; Levitt, 1978; see Barlow and Thornton, 1988). When Pro residues do occupy a position in an α-helix, it is often highly conserved across species which suggests a functional role (Barlow and Thornton, 1988). Among the membrane proteins, Pro residues are found at a significantly higher frequency in the membrane spanning region of transport proteins as compared to non-transport proteins (Brandl and Deber, 1986); it has been suggested that cis-trans isomerization of Xaa-Pro peptide bonds may be of functional importance in these molecules (Woolfson et al., 1991).

The experiments described in this paper were designed to test the effects on current activation of a Pro residue when introduced into the S4 segment of a mammalian voltage-dependent K^+ channel RBK1 (Christie et al., 1989; also called Kv1.1). Substitution of Ile^{302} or Leu^{305} by Pro within the S4 segment of RBK1, the resulting channels would have four identical mutations, did not give rise to

ionic currents. Therefore, the concatenated K^+ channel [4]RBK1, comprised of four linked RBK1 subunits, was used (Hurst et al., 1992). A Pro was introduced into the S4 segment of one or two domains of [4]RBK1, replacing either an Ile (equivalent to Ile^{302} in RBK1) or a Leu (equivalent to Leu^{305} in RBK1). Injection into *Xenopus* oocytes of mRNA encoding channels having one Pro containing S4 segment gave rise to ionic currents with altered voltage-dependence; functional channels having two such mutant S4 segments did not appear to form.

Experimental Procedures

Construction of [4]RBK1 and the mutants thereof. [4]RBK1 and the mutants thereof were constructed as previously described (Hurst et al., 1992). Briefly, RBK1 cassettes were generated by introducing unique restriction sites at the 5' and 3' ends of four RBK1 cDNAs. The restriction sites allowed the cDNAs to be ligated in frame and in a known orientation. In addition, linker regions encoding 10 Gln residues were introduced between adjacent RBK1 cassettes. In each case, the first Gln codon of the repeat replaced the natural stop codon in RBK1; the last Gln codon was followed by a restriction site encoding two additional amino acids. The ligation reactions generating [4]RBK1 'wild-type' and mutant constructs were transformed into *Escherichia coli* host strain SURE (Stratagene). Individual colonies were isolated and the plasmid DNA was analyzed by restriction digests and nucleotide sequencing.

The amino acid substitutions, Ile→Pro and Leu→Pro, were introduced at the equivalent position in either one or two domains of

[4]RBK1 using oligonucleotide directed mutagenesis. The Ile→Pro substitution was made at the position corresponding to Ile^{302} in RBK1 and the Leu→Pro substitution was made at the position corresponding to Leu^{305} in RBK1 (see below).

RBK1 S4 segment

288 L A I L R V I R L V R V F R I F K L S R H S K G L

Mutants of [4]RBK1 were made by mutating the individual RBK1 cassettes which already contained the linker regions and restriction sites. Site-directed mutants were constructed using a derivative of the pSELECT phagemid (Promega, Madison, WI) which allows rescue of the (+)-strand (JPA, unpublished results). RBK1 cassettes carrying the Ile→Pro or Leu→Pro mutation were ligated with other mutant or 'wild-type' cassettes. In some experiments, a different amino acid substitution (Tyr→Val), at the position equivalent to Tyr^{379} in RBK1, was introduced in either 'wild-type' cassettes or cassettes having the Leu→Pro substitution. The Tyr→Val substitution has previously been shown to decrease the potency of external tetraethylammonium (TEA) (Kavanaugh et al., 1991; MacKinnon and Yellen, 1990). The nucleotide sequence of all mutagenesis products was verified by DNA sequencing.

Electrophysiology and data analysis. RNA was transcribed *in vitro* using T7 RNA polymerase; 0.1 - 50 ng was injected into collagenase treated *Xenopus* oocytes (stages V-VI; see Christie et al., 1989). Membrane currents were measured 2 to 7 days following injection using either the two electrode voltage-clamp or the cut-open oocyte Vaseline-gap technique (Bezanilla et al, 1991;

Taglialatela et al., 1992). Conductance-voltage (G-V) relationships were measured using the two electrode voltage-clamp. The oocyte membrane was clamped at -80 mV and depolarized to a series of test potentials. Leak-subtracted peak currents were divided by the driving force (V_m - E_K) to obtain conductance (G), this was expressed as a fraction of its maximal value (G/G_{max}). The maximal value of conductance (G_{max}) was estimated by fitting the G-V relationship to a Boltzmann function $G/G_{max} = 1/\{1+\exp[(V_{0.5} - V_m)/k]\}$ where ($V_{0.5}$) is the potential which gave one half the maximal conductance, (V_m) is the potential of the depolarizing pulse and (k), the slope factor, is the number of mV necessary to cause an e-fold shift in conductance.

Tail currents were measured using the cut-open oocyte Vaseline gap technique (Bezanilla et al., 1991; Taglialatela et al., 1992). Oocyte membranes were clamped at -90 mV and depolarized to potentials between -60 mV and +100 mV; families of tail currents were measured at potentials between -180 and -20 mV in 120 mM symmetrical K^+. The external solution was free of Cl^- (substituted by methanesulfonate) to prevent contamination by endogenous Cl^- currents. The decaying phase of the tail currents was fitted to either a single exponential term

$$A \cdot exp(-t/\tau) \qquad (21)$$

or the sum of two exponential terms

$$A_1 \cdot exp(-t/\tau_1) + A_2 \cdot exp(-t/\tau_2) \qquad (22)$$

by least squares minimization using the Clampfit program (Axon Instruments). Requirement for a second exponential term was determined by eye.

Dose response curves were generated by clamping oocytes at -80 mV and recording currents during a 250 ms pulse to +20 mV every 30 s. TEA was applied by superfusion, and the concentration was increased cumulatively. Current amplitude was measured when it reached its new steady state in the presence of TEA. Inhibition (I, in percent) was fitted by least squares minimization to $I(\%) = 100 \cdot [TEA]/([TEA]+K)$ to provide an estimate for K, the apparent dissociation constant.

Constructs and Nomenclature.

[4]RBK1 has four linked domains, each identical to a RBK1 subunit (see "Experimental Procedures"). The individual domains of [4]RBK1 are denoted by Roman numerals I, II, III, and IV. Domains with the Leu→Pro substitution in the S4 segment are indicated by a superscript P (^{P}) (*e.g.* I^{P}-II-III-IV has four linked domains, the first of which has the Leu→Pro substitution in the S4 segment); domains with the Ile→Pro substitution are indicated with (*) (*e.g.* I-II^{*}-III-IV has four linked domains, the second of which has the Ile→Pro substitution). One additional mutation, Tyr→Val at the position equivalent to 379 in RBK1, was made in either 'wild-type' domains or domains containing the Leu→Pro substitution. Domains having the Tyr→Val substitution are indicated by a superscript V (^{V}). Domains having both the Leu→Pro and the Tyr→Val substitution are indicated with (^{P,V}).

A single construct was made with the Ile→Pro substitution in one domain (I-II*-III-IV), and a single construct was made with this substitution in two domains (I*-II*-III-IV). Three constructs were made with the Leu→Pro substitution in one domain (I^P-II-III-IV, I-II^P-III-IV, and I-II-III^P-IV). Eight different constructs were generated with the Leu→Pro in two domains. Three forms had domains with Pro containing S4 segments adjacent in the linear sequence (I^P-II^P-III-IV, I-II^P-III^P-IV and I-II-III^P-IV^P). Two forms had Pro containing domains that were separated by a 'wild-type' domain (I^P-II-III^P-IV and I-II^P-III-IV^P). Three additional constructs with two Leu→Pro substituted S4 segments were generated which had the Tyr→Val substitution in one or two domains. One had the Tyr→Val substitution in both domains with the Leu→Pro substitution ($I^{P,V}$-$II^{P,V}$-III-IV). The Tyr→Val substitution was also introduced into either one (I^V-II-III^P-IV^P) or both (I^P-II^P-III^V-IV^V) domains not having the Leu→Pro substitution.

Results

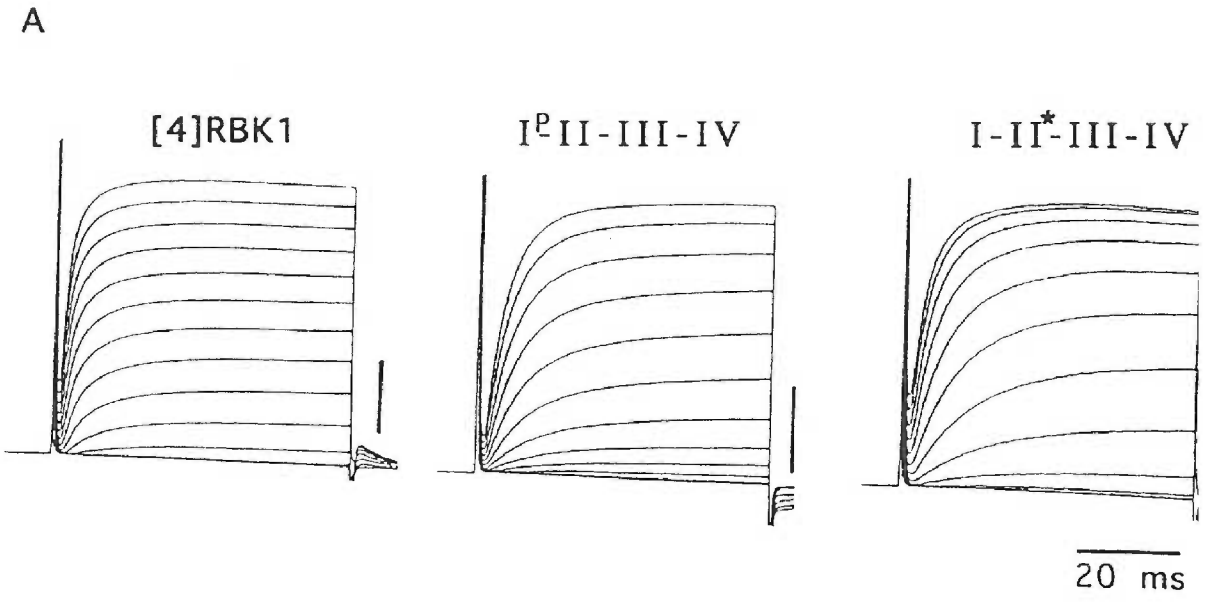
A Pro substitution in one S4 segment gave rise to voltage-dependent potassium currents with altered properties. RNA encoding [4]RBK1, and various mutant constructs thereof, was transcribed *in vitro* and injected into *Xenopus* oocytes. We have previously reported that injection of [4]RBK1 RNA into oocytes gives rise to a voltage-dependent K^+ current that is not distinguishable from RBK1 injected oocytes (Hurst et al., 1992). Injection of RNA encoding RBK1 subunits with either the Ile→Pro [RBK1(I302P)] or the

Leu→Pro [RBK1(L305P)] substitution never gave rise to ionic currents. In this case channels would have been formed by the association of four such mutant subunits.

[4]RBK1 constructs having the Leu→Pro substitution in a single S4 segment gave rise to voltage-dependent K^+ currents when RNA was injected into oocytes (Fig. 10.A). At the macroscopic level, these currents expressed less efficiently than [4]RBK1; approximately 100 - 200 times as much RNA was injected to achieve similar levels of whole cell conductance as compared to [4]RBK1 RNA. The conductance-voltage relationship of this current was shifted approximately +40 mV at the midpoint and the slope was decreased compared to [4]RBK1 (Fig. 10.B). The effect of the mutation was independent of the domain into which it was introduced, *i.e.* currents evoked from cells injected with RNA encoding I^P-II-III-IV, I-II^P-III-IV, or I-II-III^P-IV could not be distinguished. The Ile→Pro substitution introduced into one S4 segment (I-II^*-III-IV) also gave rise to a voltage-dependent K^+ current (Fig. 10.A). The conductance-voltage relation of this construct was shifted by approximately +20 mV at the midpoint but had little, if any, change in slope (Fig. 10.B). Whole cell conductance of oocytes injected with this RNA was depressed approximately 3 - 5 fold in comparison to [4]RBK1.

The deactivation kinetics of [4]RBK1 were markedly changed by introducing the Leu→Pro substitution in one S4 segment. Figure 11. shows tail currents evoked from oocytes injected with RNA encoding [4]RBK1 and I^P-II-III-IV. [4]RBK1 tail currents were well described by a single exponential term following all depolarizing

Figure 10.


A
[4]RBK1
I^P II-III-IV
I-II*-III-IV
20 ms

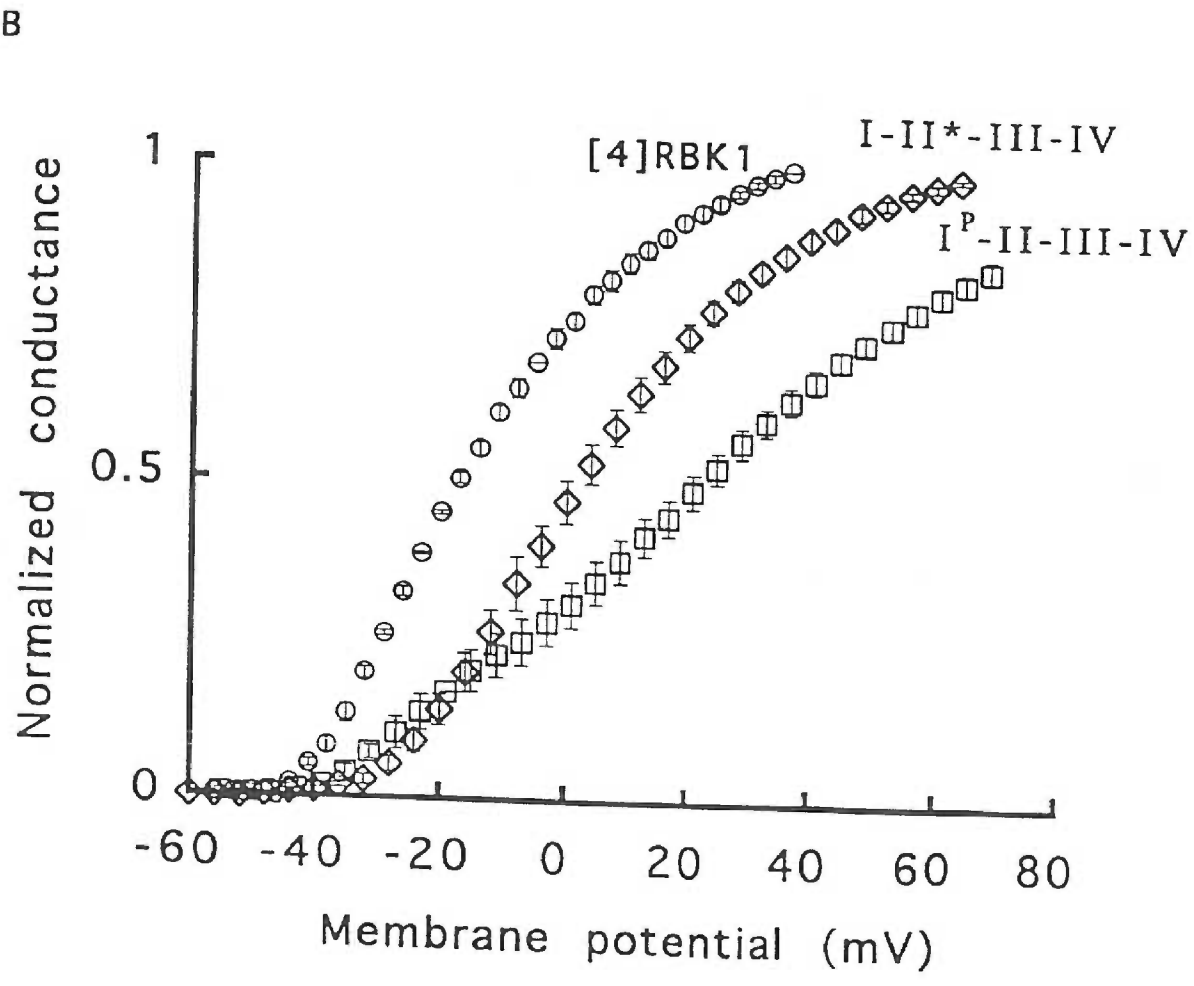
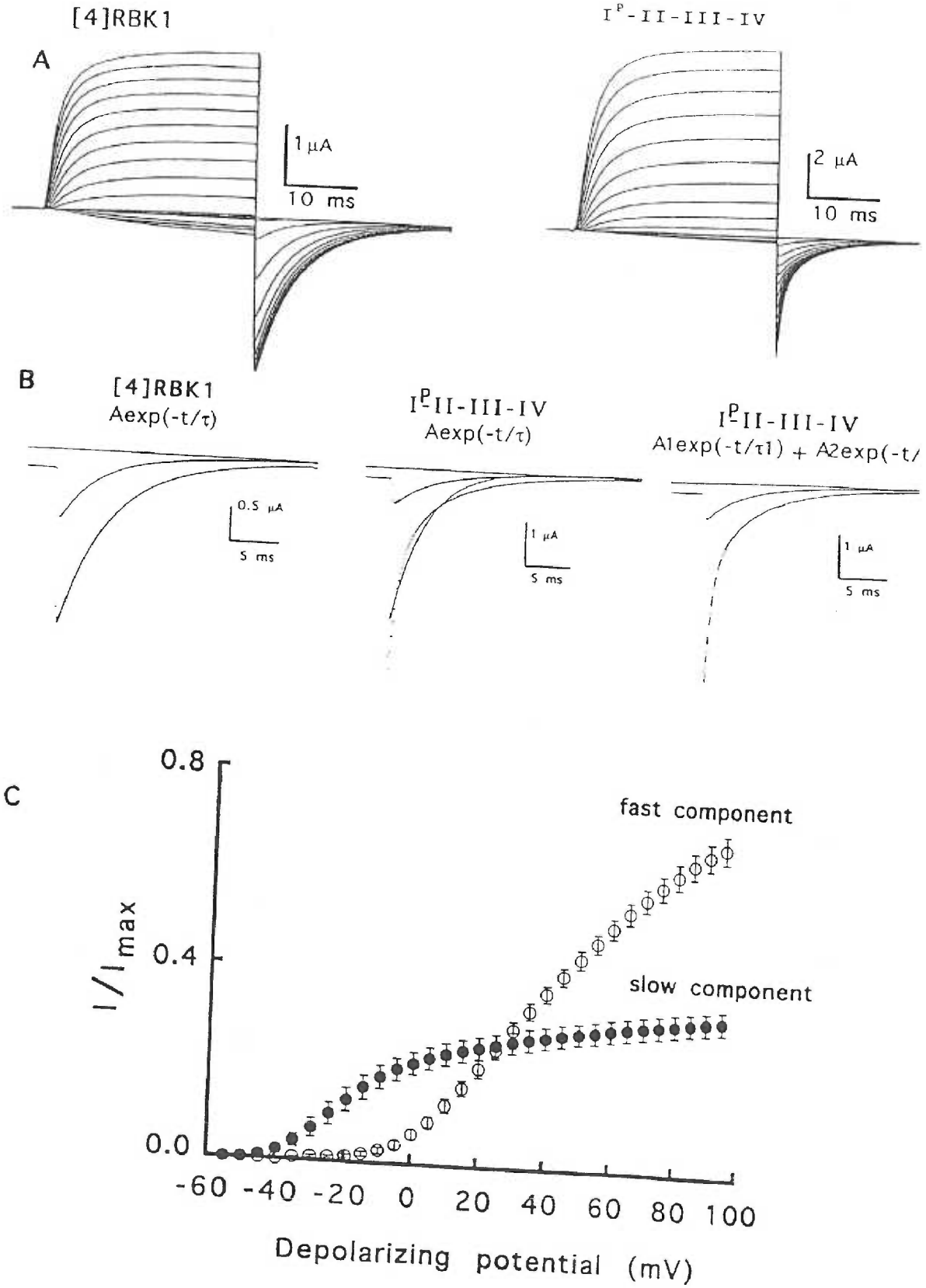

B
[4]RBK1
I-II*-III-IV
I^P-II-III-IV
Normalized conductance
1
0.5
0
-60
-40
-20
0
20
40
60
80
Membrane potential (mV)

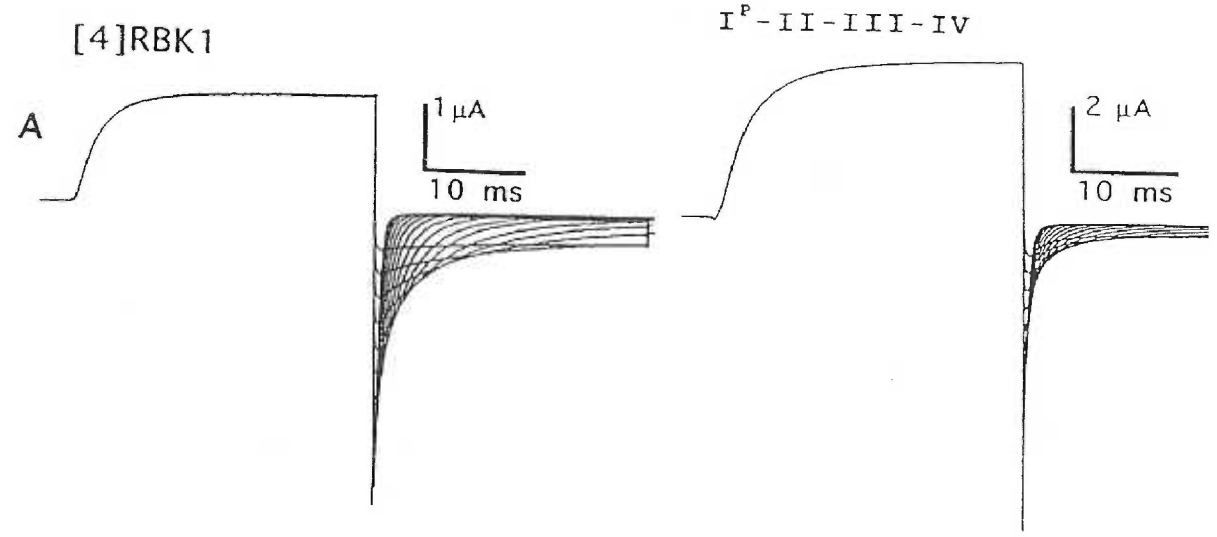
Figure 11.


[4]RBK1
A
1 μA
10 ms
I^P-II-III-IV
2 μA
10 ms
B
[4]RBK1
Aexp(-t/τ)
0.5 μA
5 ms
I^P-II-III-IV
Aexp(-t/τ)
1 μA
5 ms
I^P-II-III-IV
A1exp(-t/τ1) + A2exp(-t/
1 μA
5 ms
C
0.8
0.4
0.0
I/I max
fast component
slow component
-60 -40 -20 0 20 40 60 80 100
Depolarizing potential (mV)

pulses (Fig. 11.B). Tail currents from I^P-II-III-IV were better fitted to the sum of two exponential terms following depolarizations to potentials more positive than -20 mV (Fig. 11.B). The two components activated over different potential ranges. Figure 11.C shows the instantaneous current-voltage relationship for the fast and slow decaying components observed in oocytes injected with I^P-II-III-IV encoding RNA. The instantaneous current values were estimated by fitting the tail currents to a sum of two exponential terms and extrapolating the amplitude of each component to the instant of repolarization. The slower of the two components was observed following all depolarizations which evoked ionic currents; the decay time course of this component was not different from [4]RBK1 (Fig. 12.B). The fast decaying component was activated at more positive potentials, and the conductance was less steeply related to membrane potential (Fig. 11.C). The fast decay rate of this component was easily distinguished from that of [4]RBK1 and the slow decaying component of I^P-II-III-IV (Fig. 12.B).

Currents do not appear to pass through channels having two Pro containing S4 segments. Injection of RNA encoding constructs with the Leu→Pro substitution in two S4 segments in some cases gave rise to ionic currents. Currents were evoked when the Pro containing S4 segments were in domains adjacent in the linear sequence (*i.e.* I^P-II^P-III-IV, I-II^P-III^P-IV, and I-II-III^P-IV^P) but not when the domains were separated (*i.e.* I^P-II-III^P-IV and I-II^P-III-IV^P; Fig. 13.A). In those cases when ionic currents were evoked, the whole cell current amplitude was substantially

Figure 12.


[4]RBK1
I^P-II-III-IV
A
1 μA
10 ms
2 μA
10 ms

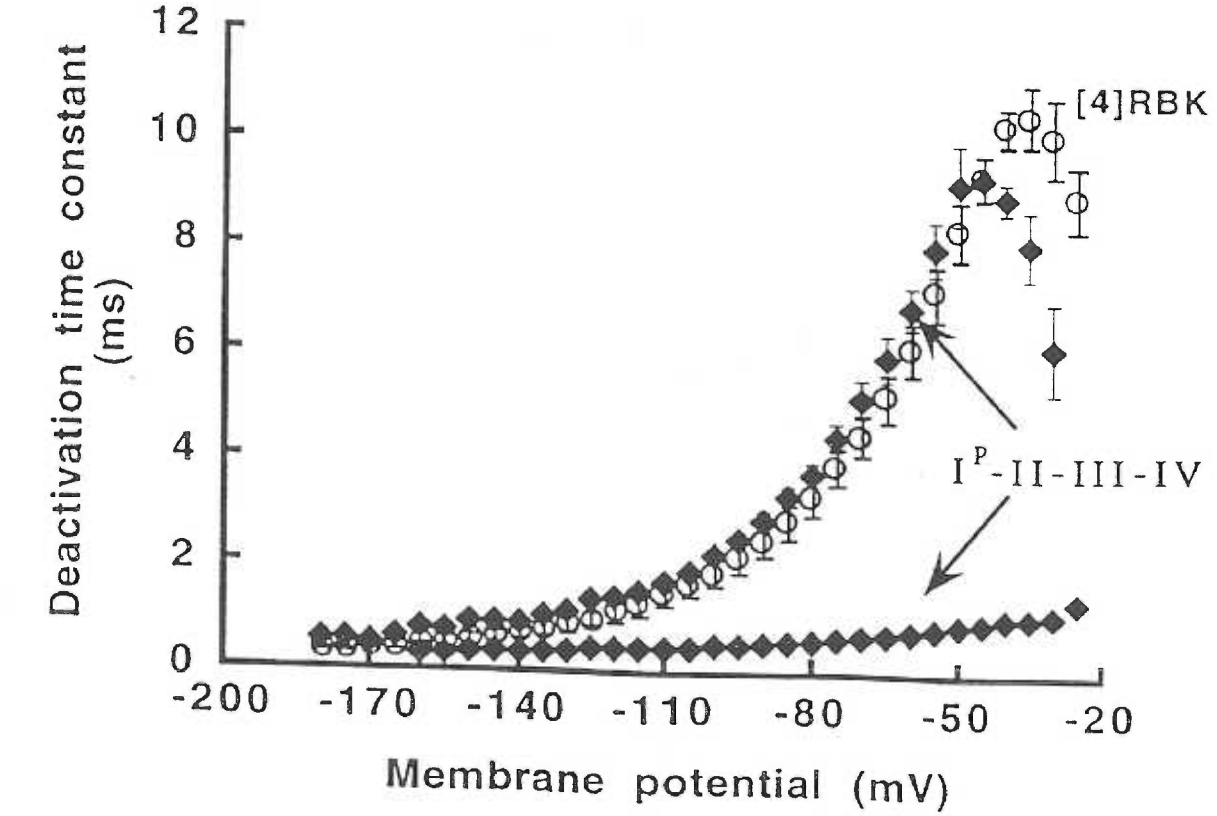
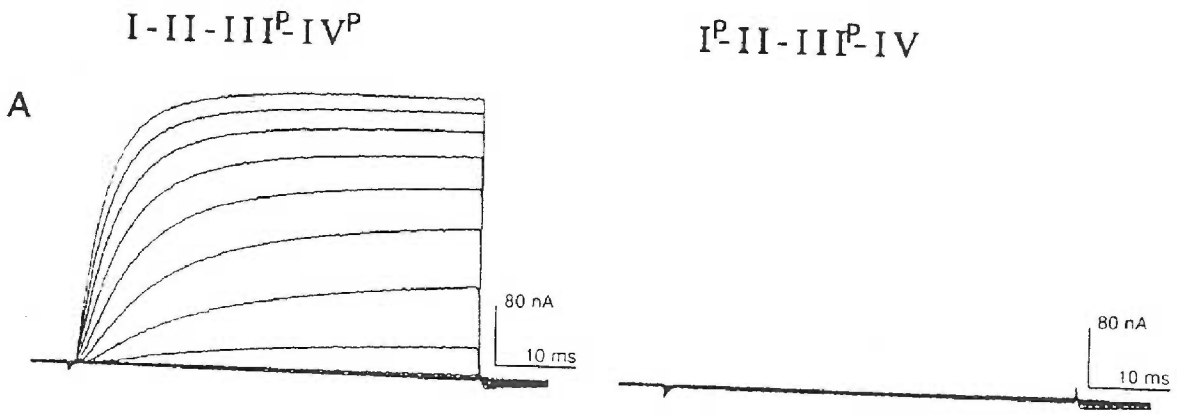
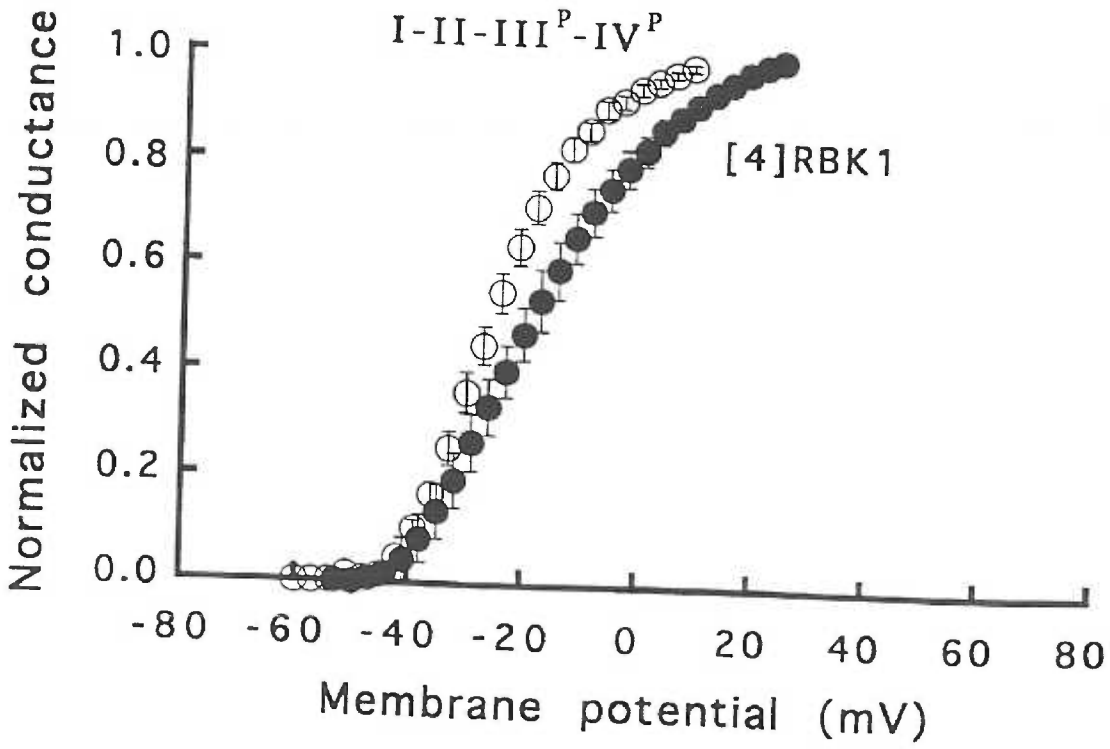

B
Deactivation time constant (ms)
12
10
8
6
4
2
0
-200
-170
-140
-110
-80
-50
-20
Membrane potential (mV)
[4]RBK
I^P-II-III-IV

Figure 13.


I-II-III^P-IV^P
I^P-II-III^P-IV
A
80 nA
10 ms
80 nA
10 ms

B

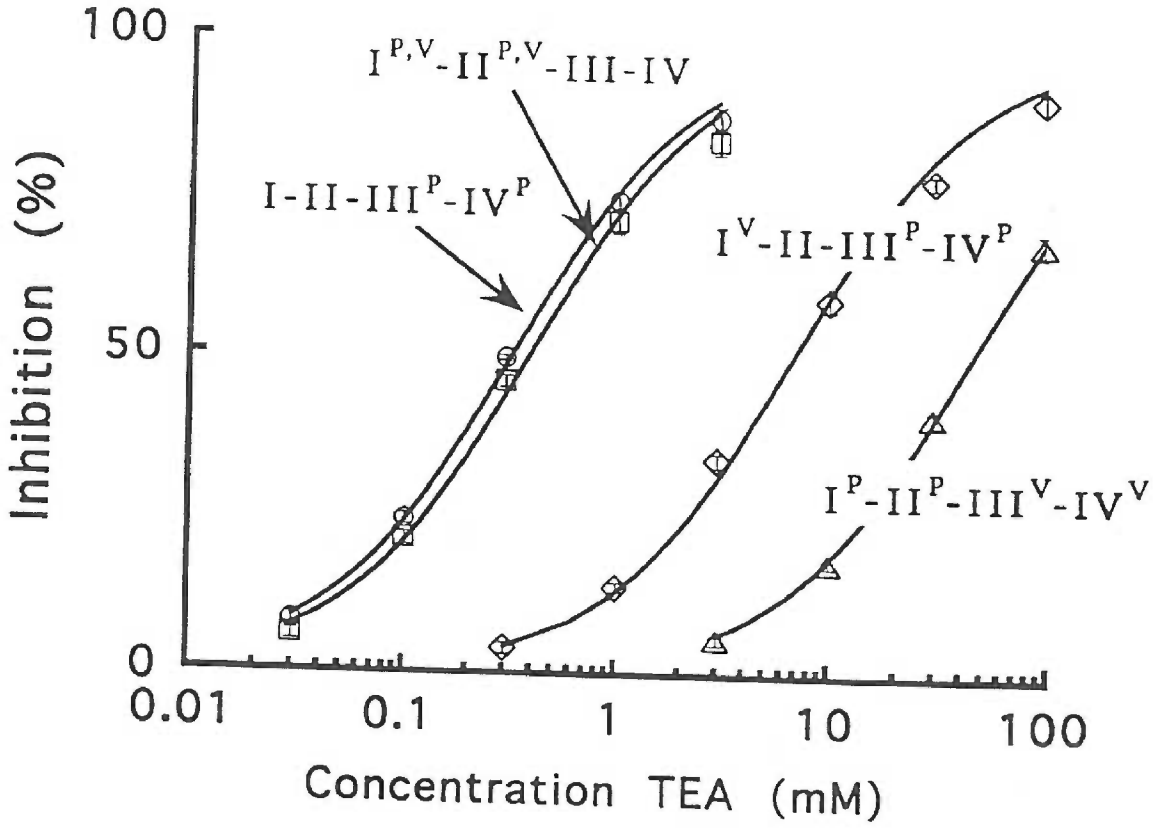

I-II-III^P-IV^P
[4]RBK1
Normalized conductance
1.0
0.8
0.6
0.4
0.2
0.0
-80
-60
-40
-20
0
20
40
60
80
Membrane potential (mV)

reduced compared to [4]RBK1, but the voltage-dependence was similar (Fig. 13.B). Approximately 1000 times as much RNA needed to be injected to achieve similar whole cell current amplitudes as [4]RBK1.

Therefore, a Tyr→Val substitution, previously shown to reduce external TEA sensitivity (Kavanaugh et al., 1991; MacKinnon and Yellen, 1990), was used to test whether the Leu→Pro substituted domains contributed to the pore of these channels. The Tyr→Val substitution was introduced into either domains having the Leu→Pro substitution or into one or both remaining 'wild-type' domains (see "Constructs and Nomenclature"). When introduced into both domains having the Leu→Pro substitution ($I^{P,V}$-$II^{P,V}$-III-IV), the Tyr→Val substitution had no effect on TEA sensitivity (Fig. 14). However, when the Tyr→Val substitution was introduced into one domain not having the Leu→Pro substitution (I^{V}-II-III^{P}-IV^{P}), TEA sensitivity was decreased approximately 20 fold. Sensitivity was further decreased when the Tyr→Val substitution was made in both domains not having the Pro substitution (I^{P}-II^{P}-III^{V}-IV^{V}). These results suggest that domains having the Leu→Pro substitution, in constructs with two such domains, did not contribute to the pore of functional channels.

Injection of RNA encoding the [4]RBK1 construct with the Ile→Pro substitution in two domains (I^{*}-II^{*}-III-IV) did give rise to ionic currents. However, as in the case of the Leu→Pro substitution, high concentrations of RNA were required to evoke measurable currents. In light of the results with the Tyr→Val substitution, this construct was not examined further.

Figure 14.


100
50
0
Inhibition (%)
$I^{P,V}$-$II^{P,V}$-III-IV
I-II-III^{P}-IV^{P}
I^{V}-II-III^{P}-IV^{P}
I^{P}-II^{P}-III^{V}-IV^{V}
0.01
0.1
1
10
100
Concentration TEA (mM)

Discussion

The voltage sensitivity of voltage-dependent ion channels is largely attributed to the S4 segments, putative α-helical transmembrane domains having a positive charge every third position. Proline residues are found at conserved positions in either one or two S4 segments in voltage-gated Na^+ or Ca^{2+} channels but are not found in the S4 segment of any *Shaker* like K^+ channel. Proline residues lack an amide proton and usually bend α-helical structures; there is some correlation between the activation kinetics of voltage-dependent ion channels and the number of proline containing S4 segments (see Guy and Conti, 1990).

Two residues (Ile^{302} and Leu^{305} in RBK1) were replaced individually by a Pro in either one or two S4 segments of the concatenated K^+ channel [4]RBK1. Channel constructs with two proline containing S4 segments probably did not form functional channels. In some cases (I^P-II^P-III-IV, I-II^P-III^P-IV, I-II-III^P-IV^P and I^*-II^*-III-IV), RNA injection into oocytes did give rise to small voltage-dependent K^+ currents; however, the Pro containing domains did not appear to contribute to the pore of these channels. This was tested for the Leu→Pro substitution using a second mutation, Tyr→Val, which decreases TEA potency. Introducing this mutation into both domains having the Leu→Pro substitution did not change TEA sensitivity; TEA potency was reduced when the equivalent substitution was made in one or both domains not having the Leu→Pro substitution (Fig. 14). We have previously reported that ≤ 5% of the channels formed following

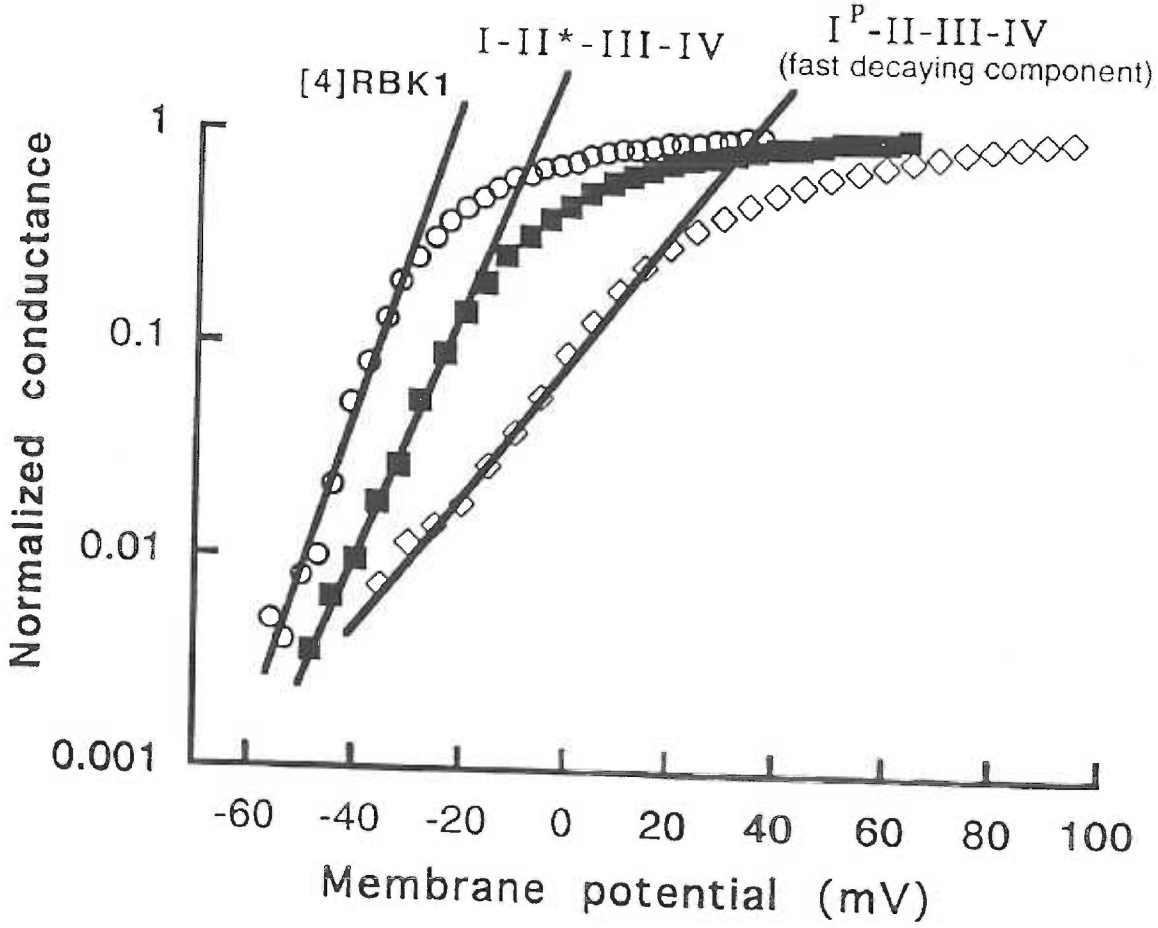
injection of [4]RBK1 RNA resulted from polymerization of either intact [4]RBK1 molecules or their degradation products (Hurst et al., 1992). The small currents (RNA concentrations had to be increased ≈1000 times in comparison of [4]RBK1 RNA to achieve similar whole cell currents) are well within the original estimate for channels forming by polymerization, and were possibly passing through such channels. It seems unlikely that these channels resulted from the polymerization of degradation products, either at the RNA or protein level, because I^{P}-II-III^{P}-IV or I-II^{P}-III-IV^{P} did not give rise to ionic currents as would be expected if individual subunits were liberated (Fig. 13.A).

Introducing a proline residue (Leu→Pro or Ile→Pro substitution) into a *single* S4 segment of [4]RBK1 gave rise to voltage-dependent K^{+} currents that were distinct from [4]RBK1. In the case of the Ile→Pro substitution, the conductance-voltage relationship was shifted approximately +20 mV without a significant change in slope (Fig. 10.B). The Leu→Pro substitution in one S4 segment shifted the conductance-voltage relationship approximately +40 mV at the midpoint; the slope of this relationship was decreased and also appeared bi-phasic (Fig. 10.B). The tail currents from oocytes injected with constructs having one Leu→Pro substitution were comprised of two exponentially decaying components. The slower of the two components decayed with a similar time course as [4]RBK1 (Fig. 12.B), and the instantaneous current-voltage relationship was similar in voltage dependence (Fig. 11.C). The fast decaying component was distinct from [4]RBK1, both in time course (Fig 12.B) and voltage dependence (Fig. 11.C).

One possibility to account for the two component decay of tail currents is that these cells expressed two different channel populations, one formed by the association of four 'wild-type' domains via polymerization. This is consistent with finding that the slow decaying component of the tail currents had a similar time course and voltage dependence as [4]RBK1. In this case, the second channel population, having a fast decay rate and shifted voltage dependence, would have the Leu→Pro substitution in one S4 segment. However, we can not exclude the possibility that the currents arose from a single population of channels having unique transition rate constants between some states, which could also account for the two component decay of the tail currents.

The single Ile→Pro substitution probably had a lesser effect on the gating process than the single Leu→Pro substitution. Figure 15. shows the conductance-voltage relationship for [4]RBK1, the singly substituted Ile→Pro construct, and the *fast* decaying component of the singly substituted Leu→Pro construct. While both Pro mutations shift the midpoint of the activation curve, only the Leu→Pro had a significant effect on the slope. A simple shift in the conductance-voltage relationship along the voltage axis, as in the case of the single Ile→Pro substitution, with little change in slope, can result from relatively small changes in the ratio of the free energies of the open and closed states. Changes in the slope of the conductance-voltage relationship, as was seen for the single Leu→Pro substitution, suggest a more radical alteration. Possibly the number of charges that cross the membrane during the voltage

Figure 15.


[4]RBK1
I-II*-III-IV
I P-II-III-IV
(fast decaying component)
Normalized conductance
1
0.1
0.01
0.001
-60
-40
-20
0
20
40
60
80
100
Membrane potential (mV)

dependent transitions was reduced or these transitions were separated over a broad potential range.

In conclusion, a Pro substitution at position 302 or 305 in one S4 segment of [4]RBK1, separated by one repeat of the charged motif, had different effects on current activation. Possibly, these positions are in structurally distinct regions of the S4 segment. Position 302 is located more central in the S4 segment than positio 305. Interestingly, a Pro substitution at position 305 appeared to have a greater effect on current activation than a Pro substitution at position 302. The decreased limiting slope caused by the Leu→Pro substitution in one S4 segment might suggest that less charge was moved during the opening of the channel (see Almers, 1978). Possibly, this mutated S4 segment contributed less, or not at all, to the activation of the current by voltage.

DISCUSSION

This dissertation describes studies of the structure and function of a rat brain voltage-dependent K^+ channel RBK1 (Christie et al., 1989; also called Kv1.1). The research focused on two aspects, the structure of the outer pore and the mechanisms of voltage-dependent activation. RBK1, a mammalian homolog of the *Shaker* gene, was isolated from a rat hippocampus complementary DNA library. Injection into *Xenopus* oocytes of *in vitro* transcribed mRNA encoding RBK1 gives rise to a voltage-dependent K^+ selective current (see Christie et al., 1989). RBK1 currents have the hallmarks of a classical delayed rectifier. In voltage-clamp experiments, the K^+ conductance of oocytes injected with RBK1 RNA increases with membrane depolarization and decreases with hyperpolarization. Currents evoked during prolonged depolarizations (>1s) only partially and slowly inactivate (time constant, 5 to 10 s; see Christie et al., 1989). These properties are characteristic of native currents found in both rat (Halliwell et al., 1986; Segal and Barker, 1984; Segal et al., 1884) and guinea pig (Gustaffson et al., 1982) hippocampal neurons.

Potassium currents modulate excitability of essentially all cells, making them a possible target for therapeutic intervention in a variety of disease states. For example, K^+ current modifiers are currently being tested and used for treatment of type II diabetes (for review, see Robertson and Steinberg, 1990), epilepsy and associated disorders (Abele and Miller, 1990), airway diseases such as asthma (Black et al., 1990; for reviews, see Barnes, 1992; Black

external mouth of the permeation pathway. Because DTX is relatively small (60 amino acids), residues on the channel that interact with DTX are likely to reside near the external mouth of the conduction pathway. Three residues, between the fifth and sixth putative transmembrane domains, were identified which influence the potency of DTX. This is consistent with the hypothesis that the outer channel mouth is comprised of amino acids in the S5-S6 loop (Guy and Conti, 1989; Durell and Guy, 1992). The combined efforts of several groups have since provided overwhelming evidence that this region of the channel peptide forms the lining to the K^+ conduction pathway (Yellen et al., 1991; Yool and Schwarz, 1991; Hartman et al., 1991).

The second and third chapters made use of a novel approach to study K^+ channel structure and function. Potassium channels are normally formed by the association of four subunits (MacKinnon, 1992). Consequently, expression of subunits having a specific mutation leads to the formation of channels with four identical mutations, one in each subunit. However, it is often of interest to know the effect of a mutation when introduced into a single or some subset of channel subunits (*e.g.* see Kavanaugh et al., 1992; Heginbotham et al., 1992; Hurst et al., 1992; Tytgat and Hess, 1992; Liman et al, 1992). Therefore, a K^+ channel comprised of four covalently joined RBK1 subunits was constructed by ligating in frame four RBK1 cDNAs. Using [4]RBK1 as a parent construct, mutations were introduced into the individual channel domains.

The second chapter of this dissertation addresses two aspects of channel structure and function. First, this chapter provides

evidence that the external tetraethylammonium (TEA) binding site is made by relatively equal contributions by four Tyr residues, one in each subunit. This finding is significant in three ways. It provides supportive evidence that functional K^+ channels naturally exist as tetramers, a hypothesis that has not been directly proven. It also suggests that the outer channel mouth is a relatively symmetrical structure. And finally, it provides some information on the dimensions of the pore at the external TEA binding site (see also Kavanaugh et al., 1992; Heginbotham and MacKinnon, 1992). That is, the pore at the TEA binding site must be sufficient narrow to allow TEA (radius = 0.4 nM) to interact with each Tyr residue. Studies such as these are an integral step in developing and refining models of K^+ channel structure; they provide specific information on the structure of the outer channel pore.

In addition, the second chapter provides evidence for cooperative interactions between domains of [4]RBK1 during the voltage-dependent opening of the pore. A conservative amino acid substitution was sequentially introduced into the four S4 segments, one into each domain. This substitution (L305I in RBK1) shifts the activation curve of RBK1 and [4]RBK1 to more depolarized potentials. Experimentally, the sequential introduction of this mutation into each S4 segment caused approximately equal shifts along the voltage axis (see also Tytgat and Hess, 1992). Hodgkin and Huxley type models of *independently* activating "gating particles" predict a marked non-linearity (1952b). This result therefore suggests that domains of [4]RBK1, and presumably the subunits of native channels, interact cooperatively during the voltage-dependent opening of the

pore. This is consistent with the observed rising phase in the time course of gating currents which suggest that the voltage-dependent conformational changes are not independent (Bezanilla et al., 1991; Taglialatela and Stefani, 1993). Most kinetic models of channel activation do not account for cooperativity (*e.g.* Hodgkin and Huxley, 1952; Zagotta and Aldrich, 1990; Koren et al., 1990). To develop more accurate descriptions of channel gating, cooperative interactions need be to quantified as has been done for other tetrameric molecules (*e.g.* Akers et al., 1992; Giudici-Orticoni, et al., 1990). One possible approach is to engineer channels in which one or more subunits are constitutively "activated". Experiments of this type may eventually allow cooperative interactions to be quantified and included into the kinetic description of channel activation.

The final chapter of this dissertation describes an initial investigation of the structure of the putative voltage-sensor (the S4 segment), and the mechanism by which it leads to the opening and closing of the pore. Voltage-dependent K^+ channel subunits are similar in predicted topology to the individual domains of voltage-dependent Na^+ and Ca^{2+} channels, but only the S4 segments show homology at the amino acid level (see Tempel et al., 1987). One feature which distinguishes Na^+ and Ca^{2+} channel S4 segments from K^+ channel S4 segments is the presence of a Pro residue. Proline residues are found at a conserved position in one or two domains of Na^+ and Ca^{2+} channels but are absent in K^+ channels. S4 segments are predicted to be α-helical in structure; a Pro residue, which lacks an amide proton, would be expected to disrupt this structure. Guy

and Conti postulated that Na^+ and Ca^{2+} channel S4 segments having a Pro residue may not contribute to the voltage-dependent opening c these channels (1990). This seems possible because these channels would have two or three non-proline containing S4 segments which could open the channel. K^+ channels on the other hand are formed as tetramers of identical or similar subunits. Indeed, no currents were evoked from oocytes injected with mutants of RBK1 in which a Pro was introduced into the S4 segment.[4] In this case, channels would have four Pro containing S4 segments. Functional voltage-dependent K^+ channels were formed when the equivalent mutation was introduced into one, but not two, domains of [4]RBK1. In one case, the voltage-dependence of current activation was reduced. Possibly, the Pro residue disrupted the function of that S4 segment, but the three remaining 'wild-type' S4 segments were sufficient to open the channel pore. One test of this hypothesis would be to measure the total charge moved per channel during the voltage-dependent opening of the pore, as has been done for other *Shaker* channels (Schoppa et al., 1992). If the proline substitution does prevent movement of that S4 segment, the gating charge per channel would be expected to be reduced by one fourth.

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[4] The proline residue substituted for the naturally occurring isoleucine at position 302 or the leucine residue at position 305 in RBK1. These are the two most probable residues to correspond to the position of the conserved proline residue in Na^+ and Ca^{2+} channels based on sequence alignments.

CONCLUDING REMARKS

In the absence of direct structural measurements, studies such as those described in this dissertation are the only means to address the structure and function of voltage-dependent K^+ channels. While no experiment is conclusive in itself, the combined efforts of many groups have provided a relatively sophisticated understanding of the mechanisms of K^+ channel function and their structure. This is an ongoing process and there are many aspects that have yet to be understood. For example, how do conformational changes in the voltage-sensor lead to the opening and closing of the channel pore? What features of the permeation pathway allow K^+ to pass 10 to 1000 times more readily than Na^+ when Na^+ is 0.4 Å smaller in radius? And what molecular mechanisms control the slower form of inactivation observed in essentially every voltage-dependent K^+ channel? These questions are of fundamental biological importance. Potassium flux across cell membranes affects every known excitable process, such as signal transduction, beat rate of the heart, secretion, and possibly learning and memory (for review, see Jan and Jan, 1989). To understand these events, and possibly to modulate them, it is necessary to understand the details of the structure and function of voltage-dependent K^+ channels.

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