POTASSIUM CHANNELS AND THE ATOMIC BASIS OF SELECTIVE ION CONDUCTION

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by

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INTRODUCTION

All living cells are surrounded by a thin, approximately 40 Å thick lipid bilayer called the cell membrane. The cell membrane holds the contents of a cell in one place so that the chemistry of life can occur, but it is a barrier to the movement of certain essential ingredients including the ions Na^+, K^+, Ca^{2+} and Cl^-. The barrier to ion flow across the membrane – known as the dielectric barrier – can be understood at an intuitive level: the cell membrane interior is an oily substance and ions are more stable in water than in oil. The energetic preference of an ion for water arises from the electric field around the ion and its interaction with neighboring molecules. Water is an electrically polarizable substance, which means that its molecules rearrange in an ion's electric field, pointing negative oxygen atoms in the direction of cations and positive hydrogen atoms toward anions. These electrically stabilizing interactions are much weaker in a less polarizable substance such as oil. Thus, an ion will tend to stay in the water on either side of a cell membrane rather than enter and cross the membrane. And yet numerous cellular processes, ranging from electrolyte transport across epithelia to electrical signal production in neurons, depend on the flow of ions across the membrane. To mediate the flow, specific protein catalysts known as ion channels exist in the cell membrane. Ion channels exhibit the following three essential properties: (1) they conduct ions rapidly, (2) many ion channels are highly selective, meaning only certain ion species flow while others are excluded, (3) their function is regulated by processes known as gating, that is, ion conduction is turned on and off in response to specific environmental stimuli. Figure 1 summarizes these properties (figure 1).

The modern history of ion channels began in 1952 when Hodgkin and Huxley published their seminal papers on the theory of the action potential in the squid giant axon (Hodgkin and Huxley, 1952a; Hodgkin and Huxley, 1952b; Hodgkin and Huxley, 1952c; Hodgkin and Huxley, 1952d). A fundamental element of their theory was that the axon membrane undergoes changes in its permeability to Na^+ and K^+ ions. The Hodgkin-Huxley theory

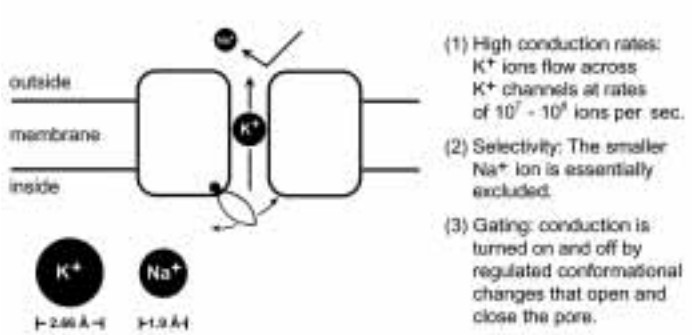

Figure 1. Ion channels exhibit three basic properties depicted in the cartoon. They conduct specific ions (for example K^+) at high rates, they are selective (a K^+ channel essentially excludes Na^+), and conduction is turned on and off by opening and closing a gate, which can be regulated by an external stimulus such as ligand-binding or membrane voltage. The relative size of K^+ and Na^+ ions is shown.

did not address the mechanism by which the membrane permeability changes occur: ions could potentially cross the membrane through channels or by a carrier-mediated mechanism. In their words 'Details of the mechanism will probably not be settled for some time' (Hodgkin and Huxley, 1952a). It is fair to say that the pursuit of this statement has accounted for much ion channel research over the past fifty years.

As early as 1955 experimental evidence for channel mediated ion flow was obtained when Hodgkin and Keynes measured the directional flow of K^+ ions across axon membranes using the isotope $^{42}K^+$ (Hodgkin and Keynes, 1955). They observed that K^+ flow in one direction across the membrane depends on flow in the opposite direction, and suggested that 'the ions should be constrained to move in single file and that there should, on average, be several ions in a channel at any moment'. Over the following two decades Armstrong and Hille used electrophysiological methods to demonstrate that Na^+ and K^+ ions cross cell membranes through unique protein pores – Na^+ channels and K^+ channels – and developed the concepts of selectivity filter for ion discrimination and gate for regulating ion flow (Hille, 1970; Hille, 1971; Hille, 1973; Armstrong, 1971; Armstrong *et al.,* 1973; Armstrong and Bezanilla, 1977; Armstrong, 1981). The patch recording technique invented by Neher and Sakmann then revealed the electrical signals from individual ion channels, as well as the extraordinary diversity of ion channels in living cells throughout nature (Neher and Sakmann, 1976).

The past twenty years have been the era of molecular biology for ion channels. The ability to manipulate amino acid sequences and express ion channels at high levels opened up entirely new possibilities for analysis. The advancement of techniques for protein structure determination and the development of synchrotron facilities also created new possibilities. For me, a scientist who became fascinated with understanding the atomic basis of life's electrical system, there could not have been a more opportune time to enter the field.

MY EARLY STUDIES: THE K^+ CHANNEL SIGNATURE SEQUENCE

The cloning of the *Shaker* K^+ channel gene from *Drosophila melanogaster* by Jan, Tanouye, and Pongs revealed for the first time a K^+ channel amino acid sequence and stimulated efforts by many laboratories to discover which of these amino acids form the pore, selectivity filter, and gate (Tempel *et al.,* 1987; Kamb *et al.,* 1987; Pongs *et al.,* 1988). At Brandeis University in Chris Miller's laboratory I had an approach to find the pore amino acids. Chris and I had just completed a study showing that charybdotoxin, a small protein from scorpion venom, inhibits a K^+ channel isolated from skeletal muscle cells by plugging the pore and obstructing the flow of ions (MacKinnon and Miller, 1988). In one of those late night 'let's see what happens if' experiments while taking a molecular biology course at Cold Spring Harbor I found that the toxin – or what turned out to be a variant of it present in the charybdotoxin preparation – inhibited the Shaker K^+ channel (MacKinnon *et al.,* 1988; Garcia *et al.,* 1994). This observation meant I could use the toxin to find the pore, and it did not take very long to identify the first site-directed mutants of the Shaker K^+ channel with altered binding of toxin (MacKinnon and Miller, 1989). I continued these experiments at Harvard Medical School where I began as assistant professor in 1989. Working with my small group at Harvard, including Tatiana Abramson, Lise Heginbotham, and Zhe Lu, and sometimes with Gary Yellen at Johns Hopkins University, we reached several interesting conclu-

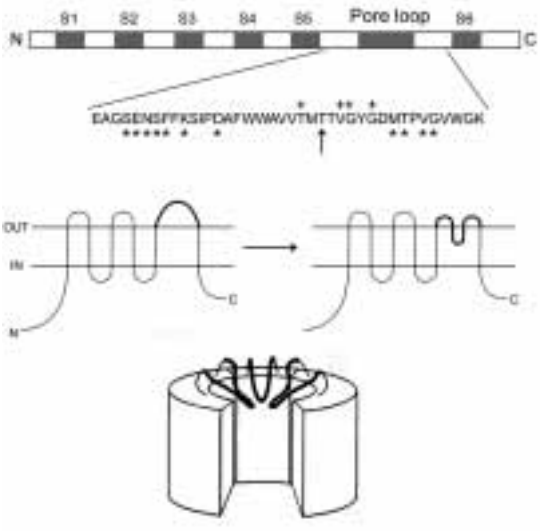

Figure 2. Early picture of a tetramer K^+ channel with a selectivity filter made of pore loops. A linear representation of a Shaker K^+ channel subunit on top shows shaded hydrophobic segments S1 to S6 and a region designated the pore loop. A partial amino acid sequence from the Shaker K^+ channel pore loop highlights amino acids shown to interact with extracellular scorpion toxins (*), intracellular tetraethylammonium (↑) and K^+ ions (+). The pore loop was proposed to reach into the membrane (middle) and form a selectivity filter at the center of four subunits (bottom).

Figure 3. The K^+ channel signature sequence shown as single letter amino acid code (blue) is highly conserved in organisms throughout the tree of life. Some K^+ channels contain six membrane-spanning segments per subunit (6TM) while others contain only two (2TM). 2TM K^+ channels correspond to 6TM K^+ channels without the first four membrane-spanning segments (S1-S4 in figure 2).

sions concerning the architecture of K^+ channels. They had to be tetramers in which four subunits encircle a central ion pathway (MacKinnon, 1991). This conclusion was not terribly surprising but the experiments and analysis to reach it gave me great pleasure since they required only simple measurements and clear reasoning with binomial statistics. We also deduced that each subunit presents a 'pore loop' to the central ion pathway (figure 2) (MacKinnon, 1995). This 'loop' formed the binding sites for scorpion toxins (MacKinnon and Miller, 1989; Hidalgo and MacKinnon, 1995; Ranganathan *et al.,* 1996) as well as the small-molecule inhibitor tetraethylammonium ion (MacKinnon and Yellen, 1990; Yellen *et al.,* 1991), which had been used by Armstrong and Hille decades earlier in their pioneering analysis of K^+ channels (Armstrong, 1971; Armstrong and Hille, 1972). Most important to my thinking, mutations of certain amino acids within the 'loop' affected the channel's ability to discriminate between K^+ and Na^+, the selectivity hallmark of K^+ channels (Heginbotham *et al.,* 1992; Heginbotham *et al.,* 1994). Meanwhile, new K^+ channel genes were discovered and they all had one obvious feature in common: the very amino acids that we had found to be important for K^+ selectivity were conserved (figure 3). We called these amino acids the K^+ channel signature sequence, and imagined four pore loops somehow forming a selectivity filter with the signature sequence amino acids inside the pore (Heginbotham *et al.,* 1994; MacKinnon, 1995).

When you consider the single channel conductance of many K^+ channels found in cells you realize just how incredible these molecular devices are. With typical cellular electrochemical gradients, K^+ ions conduct at a rate of 10^7 to 10^8 ions per second. That rate approaches the expected collision frequency of K^+ ions from solution with the entryway to the pore. This means that K^+ ions flow through the pore almost as fast as they diffuse up to it. For this to occur the energetic barriers in the channel have to be very low, something like those encountered by K^+ ions diffusing through water. All the more

remarkable, the high rates are achieved in the setting of exquisite selectivity: the K^+ channel conducts K^+, a monovalent cation of Pauling radius 1.33 Å, while essentially excluding Na^+, a monovalent cation of Pauling radius 0.95 Å. And this ion selectivity is critical to the survival of a cell. How does nature accomplish high conduction rates and high selectivity at the same time? The answer to this question would require knowing the atomic structure formed by the signature sequence amino acids, that much was clear. The conservation of the signature sequence amino acids in K^+ channels throughout the tree of life, from bacteria (Milkman, 1994) to higher eukaryotic cells, implied that nature had settled upon a very special solution to achieve rapid, selective K^+ conduction across the cell membrane. For me, this realization provided inspiration to want to directly visualize a K^+ channel and its selectivity filter.

THE KCSA STRUCTURE AND SELECTIVE K^+ CONDUCTION

I began to study crystallography, and although I had no idea how I would obtain funding for this endeavor, I have always believed that if you really want to do something then you will find a way. By happenstance I explained my plan to Torsten Wiesel, then president of Rockefeller University. He suggested that I come to Rockefeller where I would be able to concentrate on the problem. I accepted his offer and moved there in 1996. In the beginning I was joined by Declan Doyle and my wife Alice Lee MacKinnon and within a year others joined including João Morais Cabral, John Imredy, Sabine Mann and Richard Pfuetzner. We had to learn as we went along, and what we may have lacked in size and skill we more than compensated for with enthusiasm. It was a very special time. At first I did not know how we would ever reach the point of obtaining enough K^+ channel protein to attempt crystallization, but the K^+ channel signature sequence continued to appear in a growing number of prokaryotic genes, making expression in *Escherichia coli* possible. We focused our effort on a bacterial K^+ channel called KcsA from *Streptomyces lividans*, discovered by Schrempf (Schrempf *et al.,* 1995). The KcsA channel has a simple topology with only two membrane spanning segments per subunit corresponding to the Shaker K^+ channel without S1 through S4 (figure 2). Despite its prokaryotic origin KcsA closely resembled the Shaker K^+ channel's pore amino acid sequence, and even exhibited many of its pharmacological properties, including inhibition by scorpion toxins (MacKinnon *et al.,* 1998). This surprised us from an evolutionary standpoint, because why should a scorpion want to inhibit a bacterial K^+ channel! But from the utilitarian point of view of protein biophysicists we knew exactly what the scorpion toxin sensitivity meant, that KcsA had to be very similar in structure to the Shaker K^+ channel.

The KcsA channel produced crystals but they were poorly ordered and not very useful in the X-ray beam. After we struggled for quite a while I began to wonder whether some part of the channel was intrinsically disordered and interfering with crystallization. Fortunately my neighbor Brian Chait and his postdoctoral colleague Steve Cohen were experts in the analysis of soluble proteins by limited proteolysis and mass spectrometry, and their techniques

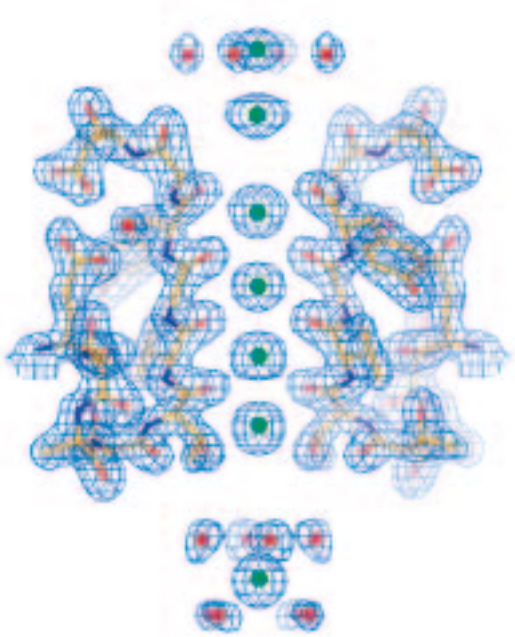

Figure 4. Electron density ($2F_o$-F_c contoured at 2 σ) from a high-resolution structure of the KcsA K^+ channel is shown as blue mesh. This region of the channel features the selectivity filter with K^+ ions and water molecules along the ion pathway. The refined atomic model is shown in the electron density. Adapted from (Zhou *et al.,* 2001b).

applied beautifully to a membrane protein. We found that KcsA was as solid as a rock, except for its C-terminus. After removing disordered amino acids from the c-terminus with chymotrypsin the crystals improved dramatically, and we were able to solve an initial structure at a resolution of 3.2 Å (Doyle *et al.,* 1998). We could not clearly see K^+ in the pore at this resolution, but my years of work on K^+ channel function told me that Rb^+ and Cs^+ should be valuable electron dense substitutes for K^+, and they were. Rubidium and Cs^+ difference Fourier maps showed these ions lined up in the pore – as Hodgkin and Keynes might have imagined in 1955 (Hodgkin and Keynes, 1955).

The KcsA structure was altogether illuminating, but before I describe it, I will depart from chronology to explain the next important technical step. A very accurate description of the ion coordination chemistry inside the selectivity filter would require a higher resolution structure. With 3.2 Å data we could infer the positions of the main-chain carbonyl oxygen atoms by applying our knowledge of small molecule structures, that is our chemical intuition, but we needed to see the selectivity filter atoms in detail. A high-resolution structure was actually quite difficult to obtain. After more than three additional years of work by João and then Yufeng (Fenny) Zhou we finally managed to produce high-quality crystals by attaching monoclonal Fab fragments to KcsA. These crystals provided the information we needed, a structure at a resolution of 2.0 Å in which K^+ ions could be visualized in the grasp of selec-

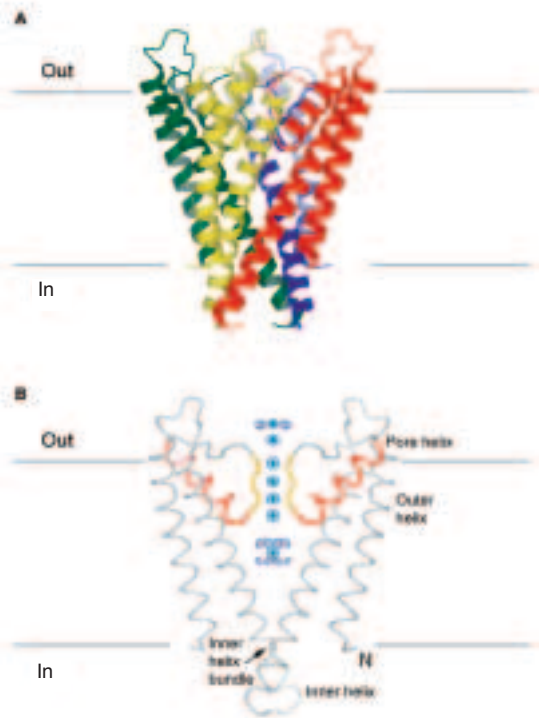

Figure 5. (A) A ribbon representation of the KcsA K^+ channel with its four subunits colored uniquely. The channel is oriented with the extracellular solution on top. (B) The KcsA K^+ channel with front and back subunits removed, colored to highlight the pore-helices (red) and selectivity filter (yellow). Electron density in blue mesh is shown along the ion pathway. Labels identify the pore, outer, and inner helices and the inner helix bundle. The outer and inner helices correspond to S5 and S6 in figure 2.

tivity filter protein atoms (figure 4) (Zhou *et al.,* 2001b). What did the K^+ channel structure tell us and why did nature conserve the K^+ channel signature sequence amino acids?

Not all protein structures speak to you in an understandable language, but the KcsA K^+ channel does. Four subunits surround a central ion pathway that crosses the membrane (figure 5A). Two of the four subunits are shown in figure 5B with electron density from K^+ ions and water along the pore. Near the center of the membrane the ion pathway is very wide, forming a cavity about 10 Å in diameter with a hydrated K^+ ion at its center. Each subunit directs the C-terminal end of a 'pore helix', shown in red, toward the ion. The C-terminal end of an α-helix is associated with a negative 'end charge' due to carbonyl oxygen atoms that do not participate in secondary structure hydrogen bonding, so the pore helices are directed as if to stabilize the K^+ ion in the cavity. At the beginning of this lecture I raised the fundamental issue of the cell membrane being an energetic barrier to ion flow because of its oily interior. KcsA allows us to intuit a simple logic encoded in its structure, and electrostatic calculations support the intuition (Roux and MacKinnon, 1999): the K^+ channel lowers the membrane dielectric barrier by hydrating a K^+ ion deep inside the membrane, and by stabilizing it with α-helix end charges.

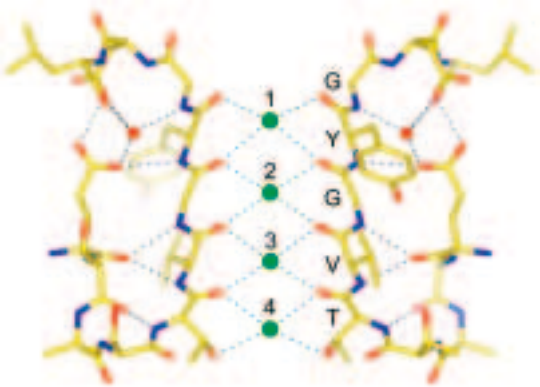

Figure 6. Detailed structure of the K^+ selectivity filter (two subunits). Oxygen atoms coordinate K^+ ions (green spheres) at positions 1 to 4 from the extracellular side. Single letter amino acid code identifies select signature sequence amino acids. Yellow, blue and red correspond to carbon, nitrogen and oxygen atoms, respectively. Green and gray dashed lines show oxygen-K^+ and hydrogen bonding interactions.

How does the K^+ channel distinguish K^+ from Na^+? Our earlier mutagenesis studies had indicated that the signature sequence amino acids would be responsible for this most basic function of a K^+ channel. Figure 6 shows the structure formed by the signature sequence – the selectivity filter – located in the extracellular third of the ion pathway. The glycine amino acids in the sequence TVGYG have dihedral angles in or near the left-handed helical region of the Ramachandran plot, as does the threonine, allowing the main-chain carbonyl oxygen atoms to point in one direction, toward the ions along the pore. It is easy to understand why this sequence is so conserved among K^+ channels: the alternating glycine amino acids permit the required dihedral angles, the threonine hydroxyl oxygen atom coordinates a K^+ ion, and the side-chains of valine and tyrosine are directed into the protein core surrounding the filter to impose geometric constraint. The end result when the subunits come together is a narrow tube consisting of four equal spaced K^+ binding sites, labeled 1 to 4 from the extracellular side. Each binding site is a cage formed by eight oxygen atoms on the vertices of a cube, or a twisted cube called a square antiprism (figure 7). The binding sites are very similar to the single alkali metal site in nonactin, a K^+ selective antibiotic with nearly identical K^+-oxygen distances (Dobler *et al.,* 1969; Dunitz and Dobler, 1977). The principle of K^+ selectivity is implied in a subtle feature of the KcsA crystal structure. The oxygen atoms surrounding K^+ ions in the selectivity filter are arranged quite like the water molecules surrounding the hydrated K^+ ion in the cavity. This comparison conveys a visual impression of binding sites in the filter paying for the energetic cost of K^+ dehydration. The Na^+ ion is apparently too small for these K^+-sized binding sites, so its dehydration energy is not compensated.

The question that compelled us most after seeing the structure was exactly

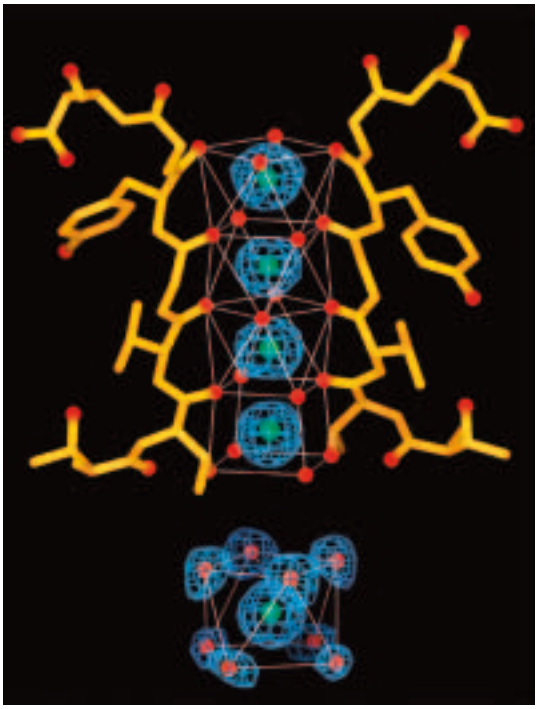

Figure 7. A K^+ channel mimics the hydration shell surrounding a K^+ ion. Electron density (blue mesh) for K^+ ions in the filter and for a K^+ ion and water molecules in the central cavity are shown. White lines highlight the coordination geometry of K^+ in the filter and in water. Adapted from (Zhou *et al.,* 2001b).

how many ions are in the selectivity filter at a given time? To begin to understand how ions move through the filter we needed to know the stoichiometry of the ion conduction reaction, and that meant knowing how many ions can occupy the filter. Four binding sites were apparent, but are they all occupied at once? Four K^+ ions in a row separated by an average center-to-center distance of 3.3 Å seemed unlikely for electrostatic reasons. From an early stage we suspected that the correct number would be closer to two, because two ions more easily explained the electron density we observed for the larger alkali metal cations Rb^+ and Cs^+ (Doyle *et al.,* 1998; Morais-Cabral *et al.,* 2001). Quantitative evidence for the precise number of ions came with the high-resolution structure and with the analysis of Tl^+ (Zhou and MacKinnon, 2003). Thallium is the most ideally suited 'K^+ analog' because it flows through K^+ channels, has a radius and dehydration energy very close to K^+, and has the favorable crystallographic attributes of high electron density and an anomalous signal. The one serious difficulty in working with Tl^+ is its insolubility with Cl^-. Fenny meticulously worked out the experimental conditions and determined that on average there are between two and two and a half conducting ions in the filter at once, with an occupancy at each position around one half.

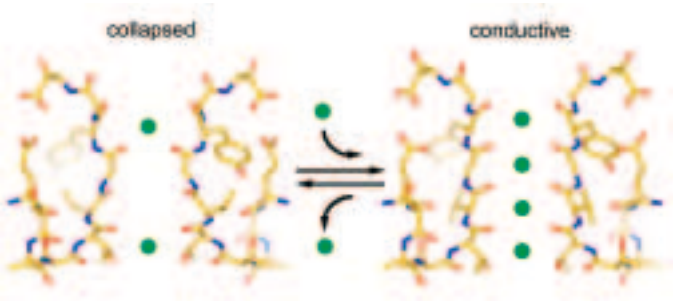

Figure 8. The selectivity filter can adopt two conformations. At low concentrations of K^+ on average one K^+ ion resides at either of two sites near the ends of the filter, which is collapsed in the middle. At high concentrations of K^+ a second ion enters the filter as it changes to a conductive conformation. On average, two K^+ ions in the conductive filter reside at four sites, each with about half occupancy.

We also observed that if the concentration of K^+ (or Tl^+) bathing the crystals is lowered sufficiently (below normal intracellular levels) then a reduction in the number of ions from two to one occurs and is associated with a structural change to a 'collapsed' filter conformation, which is pinched closed in the middle (Zhou *et al.,* 2001b; Zhou and MacKinnon, 2003). At concentrations above 20 mM the entry of a second K^+ ion drives the filter to a 'conductive' conformation, as shown in figure 8. Sodium on the other hand does not drive the filter to a 'conductive' conformation even at concentrations up to 500 mM.

The K^+-induced conformational change has thermodynamic consequences for the affinity of two K^+ ions in the 'conductive' filter. It implies that a fraction of the second ion's binding energy must be expended as work to bring about the filter's conformational change, and as a result the two ions will bind with reduced affinity. To understand this statement at an intuitive level, recognize that for two ions to reside in the filter they must oppose its tendency to collapse and force one of them out, i.e. the two-ion 'conductive' conformation is under some tension, which will tend to lower K^+ affinity. This is a desirable property for an ion channel because weak binding favors high conduction rates. The same principle, referred to as the 'induced fit' hypothesis, had been proposed decades earlier by enzymologists to explain high specificity with low substrate affinity in enzyme catalysis (Jencks, 1987).

In the 'conductive' filter if two K^+ ions were randomly distributed then they would occupy four sites in six possible ways. But several lines of evidence hinted to us that the ion positions are not random. For example Rb^+ and Cs^+ exhibit preferred positions with obviously low occupancy at position 2 (Morais-Cabral *et al.,* 2001; Zhou and MacKinnon, 2003). In K^+ we observed an unusual doublet peak of electron density at the extracellular entryway to the selectivity filter, shown in figure 9 (Zhou *et al.,* 2001b). We could explain this density if K^+ is attracted from solution by the negative protein surface charge near the entryway and at the same time repelled by K^+ ions inside the filter. Two discrete peaks implied two distributions of ions in the filter. If K^+ ions

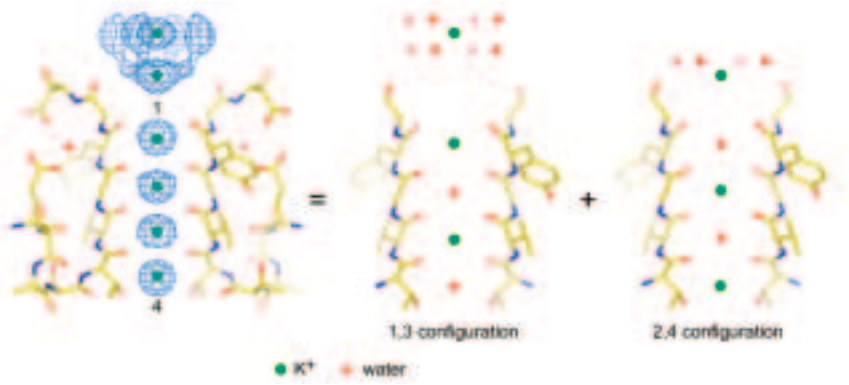

Figure 9. Two K^+ ions in the selectivity filter are hypothesized to exist predominantly in two specific configurations 1,3 and 2,4 as shown. K^+ ions and water molecules are shown as green and red spheres, respectively. Adapted from (Zhou *et al.,* 2001b).

tend to be separated by a water molecule for electrostatic reasons then the two dominant configurations would be 1,3 (K^+ ions in positions 1 and 3 with a water molecule in between) and 2,4 (K^+ ions in positions 2 and 4 with a water molecule in between). A mutation at position 4 (threonine to cysteine) was recently shown to influence K^+ occupancy at positions 2 and 4 but not at 1 and 3, providing strong evidence for specific 1,3 and 2,4 configurations of K^+ ions inside the selectivity filter (Zhou and MacKinnon, 2004).

Discrete configurations of an ion pair suggested a mechanism for ion conduction (figure 10A) (Morais-Cabral *et al.,* 2001). The K^+ ion pair could diffuse back and forth between 1,3 and 2,4 configurations (bottom pathway), or alternatively an ion could enter the filter from one side of the membrane as the ion-water queue moves and a K^+ exits at the opposite side (the top pathway). Movements would have to be concerted because the filter is no wider than a K^+ ion or water molecule. The two paths complete a cycle: in one complete cycle each ion moves only a fraction of the total distance through the filter, but the overall electrical effect is to move one charge all the way. Because two K^+ ions are present in the filter throughout the cycle we expect there should be electrostatic repulsion between them. Together with the filter conformational change that is required to achieve a 'conductive' filter with two K^+ ions in it, electrostatic repulsion should favor high conduction rates by lowering K^+ affinity.

Absolute rates from 10^7 to 10^8 ions per second are truly impressive for a highly selective ion channel. One aspect of the crystallographic data suggests that very high conductance K^+ channels such as KcsA might operate near the maximum rate that the conduction mechanism will allow. All four positions in the filter have a K^+ occupancy close to one half, which implies that the 1,3 and 2,4 configurations are equally probable, or energetically equivalent, but there is no *a priori* reason why this should be. A simulation of ions diffusing around the cycle offers a possible explanation: maximum flux is achieved when the energy difference between the 1,3 and 2,4 configurations is zero be-

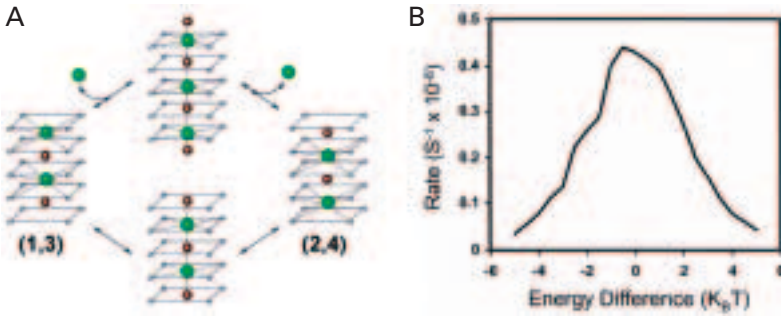

Figure 10. (A) Through-put cycle for K^+ conduction invoking 1,3 and 2,4 configurations. The selectivity filter is represented as five square planes of oxygen atoms. K^+ and water are shown as green and red spheres, respectively. (B) Simulated K^+ flux around the cycle is graphed as a function of the energy difference between the 1,3 and 2,4 configurations. Adapted from (Morais-Cabral *et al.,* 2001).

cause that is the condition under which the 'energy landscape' for the conduction cycle is smoothest (figure 10B). The energetic balance between the configurations therefore might reflect the optimization of conduction rate by natural selection (Morais-Cabral *et al.,* 2001). It is not so easy to demonstrate this point experimentally but it is certainly fascinating to ponder.

COMMON STRUCTURAL PRINCIPLES UNDERLIE K^+ AND Cl^- SELECTIVITY

The focus of this lecture is K^+ channels, but for a brief interlude I would like to show you a Cl^- selective transport protein. By comparing a K^+ channel and a Cl^- 'channel' we can begin to appreciate familiar themes in nature's solutions to different problems: getting cations and anions across the cell membrane. ClC Cl^- channels are found in many different cell types and are associated with a number of physiological processes that require Cl^- ion flow across lipid membranes (Jentsch *et al.,* 1999; Maduke *et al.,* 2000). As is the case for K^+ channels, ClC family genes are abundant in prokaryotes, a fortunate circumstance for protein expression and structural analysis. When Raimund Dutzler joined my laboratory he, Ernest Campbell and I set out to address the structural basis of Cl^- ion selectivity. We determined crystal structures of two bacterial members of the ClC Cl^- channel family, one from *Escherichia coli* (EcClC) and another from *Salmonella typhimurium* (StClC) (Dutzler *et al.,* 2002). Recent studies by Miller on the function of EcClC have shown that it is actually a Cl^- – proton exchanger (Accardi and Miller, 2004). We do not yet know why certain members of this family of Cl^- transport proteins function as channels and others as exchangers, but the crystal structures are fascinating and give us a view of Cl^- selectivity. Architecturally the ClC proteins are unrelated to K^+ channels, but if we focus on the ion pathway certain features are similar (figure 11). As we saw in K^+ channels, the ClC proteins have α-helices pointed at the ion pathway, but the direction is reversed with the positive

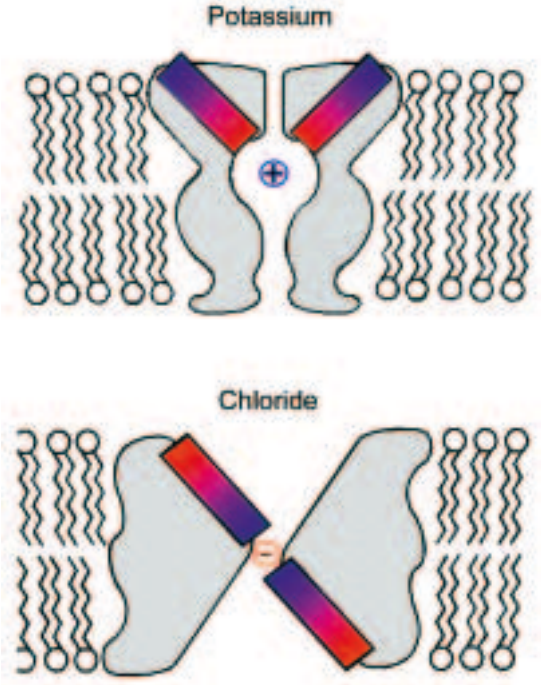

Figure 11. The overall architecture of K^+ channels and ClC Cl^- transport proteins is very different but certain general features are similar. One similarity shown here is the use of α-helix end charges directed toward the ion pathway. The negative C-terminal end charge (red) points to K^+. The positive N-terminal end charge (blue) points to Cl^-.

charge of the N-terminus close to Cl^-. This makes perfect sense for lowering the dielectric barrier for a Cl^- ion. In ClC we see that ions in its selectivity filter tend to be coordinated by main chain protein atoms, with amide nitrogen atoms surrounding Cl^- instead of carbonyl oxygen atoms surrounding K^+ (figure 12). We also see that both the K^+ and Cl^- selectivity filters contain multiple close-spaced binding sites and appear to contain more than one ion, perhaps to exploit electrostatic repulsion between ions in the pore. I find these similarities fascinating. They tell us that certain basic physical principles are important, such as the use of α-helix end charges to lower the dielectric barrier when ions cross the lipid membrane.

TRYING TO SEE A K^+ CHANNEL OPEN AND CLOSE

Most ion channels conduct when called upon by a specific stimulus such as the binding of a ligand or a change in membrane voltage (Hille, 2001). The processes by which ion conduction is turned on are called gating. The conduction of ions occurs on a time scale that is far too rapid to involve very large protein conformational changes. That is undoubtedly one of the reasons why a single KcsA structure could tell us so much about ion selectivity and con-

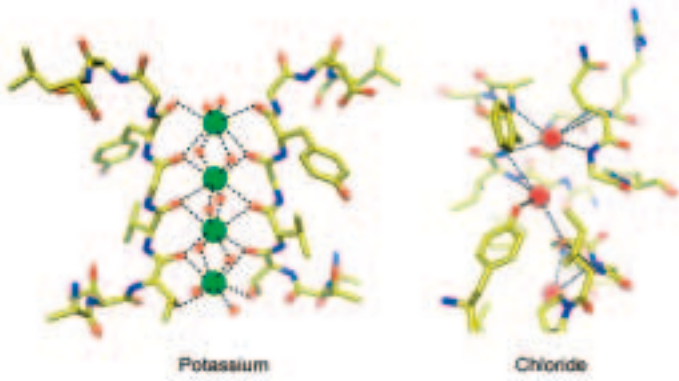

Figure 12. K^+ and Cl^- selectivity filters make use of main chain atoms to coordinate ions: carbonyl oxygen atoms for K^+ ions (green spheres) and amide nitrogen atoms for Cl^- ions (red spheres). Both filters contain multiple close-spaced ion binding sites. The Cl^- selectivity filter is that of a mutant ClC in which a glutamate amino acid was changed to glutamine (Dutzler *et al.,* 2003).

duction. Gating on the other hand occurs on a much slower time scale and can involve large protein conformational changes. The challenge for a structural description of gating is to capture a channel in both opened (on) and closed (off) conformations so that they can be compared.

In the KcsA K^+ channel gating is controlled by intracellular pH and lipid membrane composition, but unfortunately the KcsA channel's open probability reaches a maximum value of only a few percent in functional assays (Cuello *et al.,* 1998; Heginbotham *et al.,* 1998). At first we had no definitive way to know whether a gate was open or closed in the crystal structures. In the 1970s Armstrong had proposed the existence of a gate near the intracellular side of the membrane in voltage dependent K^+ channels because he could 'trap' large organic cations inside the pore between a selectivity filter near the extracellular side and a gate near the intracellular side (Armstrong, 1971; Armstrong, 1974). Following these ideas we crystallized KcsA with a heavy atom version of one of his organic cations, tetrabutyl antimony (TBA), and found that it binds inside the central cavity of KcsA (Zhou *et al.,* 2001a). This was very interesting because the ~10 Å diameter of TBA far exceeds the pore diameter leading up to the cavity: in KcsA the intracellular pore entryway is constricted to about 3.5 Å by the inner helix bundle (figure 5B). Seeing TBA 'trapped' in the cavity behind the inner helix bundle evoked Armstrong's classical view of K^+ channel gating, and implied that the inner helix bundle serves as a gate and is closed in KcsA. Mutational and spectroscopic studies in other laboratories also pointed to the inner helix bundle as a possible gate-forming structural element (Perozo *et al.,* 1999; del Camino *et al.,* 2000).

Youxing Jiang and I hoped we could learn more about K^+ channel gating by determining the structures of new K^+ channels. From gene sequence analysis we noticed that many prokaryotic K^+ channels contain a large C-ter-

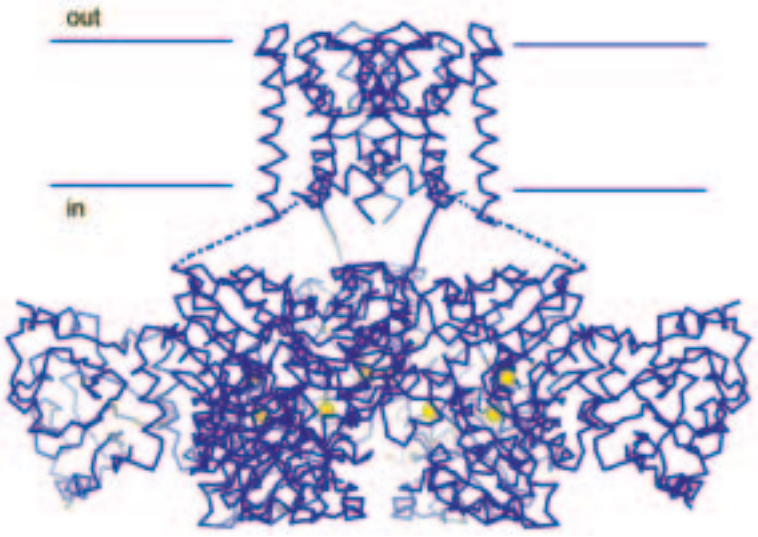

Figure 13. The MthK K^+ channel contains an intracellular gating ring (bottom) attached to its ion conduction pore (top). Ca^{2+} ions (yellow spheres) are bound to the gating ring in clefts in between domains. The connections between the gating ring and the pore, which were poorly ordered in the crystal, are shown as dashed lines.

minus that encodes what we called RCK domains and we suspected that these domains control pore opening, perhaps through binding of an ion or a small-molecule. Initally we determined the structure of isolated RCK domains from an *Escherichia coli* K^+ channel, but by themselves they were not very informative beyond hinting that a similar structure exists on the C-terminus of eukaryotic Ca^{2+}-dependent 'BK' channels (Jiang *et al.,* 2001). We subsequently determined the crystal structure of MthK, complete K^+ channel containing RCK domains, from *Methanobacterium thermoautotrophicus* (figure 13) (Jiang *et al.,* 2002a). This structure was extremely informative. The RCK domains form a 'gating ring' on the intracellular side of the pore. In clefts between domains we could see what appeared to be divalent cation binding sites, and the crystals had been grown in the presence of Ca^{2+}. In functional assays we discovered that the open probability of the MthK channel increased as Ca^{2+} or Mg^{2+} concentration was raised, giving us good reason to believe that the crystal structure should represent the open conformation of a K^+ channel.

In our MthK structure the inner helix bundle is opened like the aperture of a camera (figure 14) (Jiang *et al.,* 2002b). As a result, the pathway leading up to the selectivity filter from the intracellular side is about 10 Å wide, explaining how Armstrong's large organic cations can enter the cavity to block a K^+ channel, and how K^+ ions gain free access to the selectivity filter through aqueous diffusion. By comparing the KcsA and MthK channel structures it seemed that we were looking at examples of closed and opened K^+ channels, and could easily imagine the pore undergoing a conformational change from closed to open. To open, the inner helices would have to bend at a point halfway across

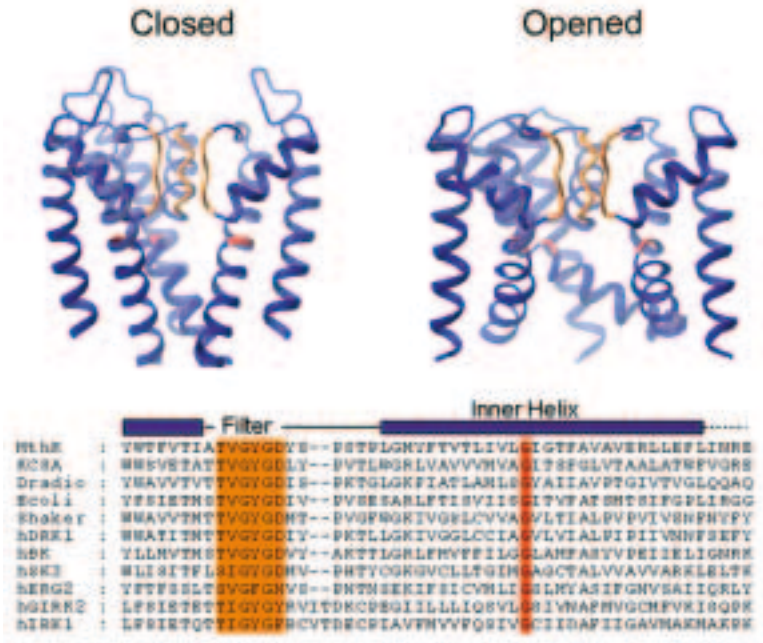

Figure 14. KcsA and MthK represent closed and opened K^+ channels. Three subunits of the closed KcsA K^+ channel (left) and opened MthK K^+ channel (right) are shown. The inner helices of MthK are bent at a glycine gating hinge (red), allowing the inner helix bundle to open. Partial amino acid sequences from a variety of K^+ channels with different gating domains are compared. Colors highlighting the selectivity filter sequence (gold) and inner helix glycine hinge (red) match colors used in the structures. Adapted from (Jiang *et al.,* 2002b).

the membrane as their C-terminus is displaced laterally away from the pore axis by conformational changes in the gating ring. A glycine amino acid facilitates the bending in MthK by introducing a hinge point in the middle of the inner helix. Like MthK, KcsA and many other K^+ channels contain a glycine at the very same location; its conservation suggests that the inner helices move in a somewhat similar manner in many different K^+ channels (figure 14).

Gating domains convert a stimulus into pore opening. Further studies are needed to understand how the free energy of Ca^{2+} binding is converted into pore opening in the MthK channel. And the mechanistic details of ligand gating will vary from one channel type to the next because nature is very modular with ion channels, just like with other proteins. Gene sequences show us that a multitude of different domains can be found attached to the inner helices of different K^+ channels, allowing ions such as Ca^{2+} or Na^+, small organic molecules, and even regulatory proteins to control the conformational state of the pore and so gate the ion channel (http://www.ncbi.nlm.nih.gov/BLAST/) (Atkinson *et al.,* 1991; Schumacher *et al.,* 2001; Yuan *et al.,* 2003; Kubo *et al.,* 1993).

A fundamentally different kind of gating domain allows certain K^+, Na^+, Ca^{2+} and nonselective cation channels to open in response to membrane volt-

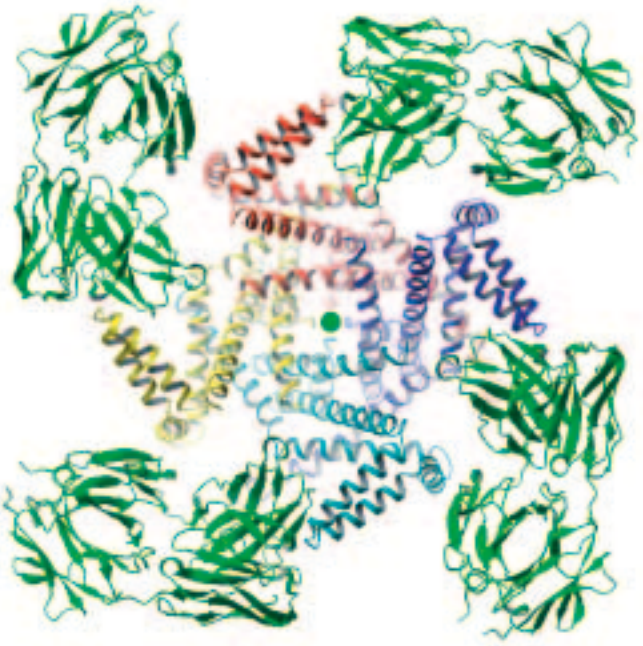

Figure 15. Crystal structure of the KvAP K^+ channel in complex with monoclonal antibody Fab fragments. The channel is viewed along the pore axis from the intracellular side of the membrane, with α-helical subunits colored in blue, yellow, cyan, and red. One Fab fragment (green) is bound to the helix-turn-helix element of the voltage sensor on each subunit. From (Jiang *et al.,* 2003a).

age changes. Referred to as voltage sensors, these domains are connected to the outer helices of the pore and form a structural unit within the membrane. The basic principle of operation for a voltage sensor is the movement of protein charges through the membrane electric field coupled to pore opening (Armstrong and Bezanilla, 1974; Sigworth, 1994; Bezanilla, 2000). Like transistors in an electronic device, voltage-dependent channels are electrical switches. They are a serious challenge for crystallographic analysis because of their conformational flexibility. Youxing Jiang and I working with Alice Lee and Jiayun Chen solved the structure of a voltage-dependent K^+ channel, KvAP, from the thermophilic Archea *Aeropyrum pernix* (Ruta *et al.,* 2003; Jiang *et al.,* 2003a) (figure 15). In the crystal of KvAP the voltage sensors, held by monoclonal Fab fragments, adopted a non-native conformation. This observation in itself is meaningful as it underscores the intrinsic flexibility of voltage sensors: in contrast Fab fragments had little effect on the more rigid KcsA K^+ channel and ClC Cl^- channel homolog, both of which we determined in the presence and absence of Fab fragments (Doyle *et al.,* 1998; Zhou *et al.,* 2001b; Dutzler *et al.,* 2002; Dutzler *et al.,* 2003). KvAP's voltage sensors contain a hydrophobic helix-turn-helix element with arginine residues beside the pore (Jiang *et al.,* 2003a), and functional experiments using tethered bi-

otin and avidin show that this element moves relative to the plane of the membrane (Jiang *et al.,* 2003b). Additional structures revealing different channel conformations will be needed to better understand the mechanistic details of voltage-dependent gating. But the KvAP structure and associated functional studies have provided a conceptual model for voltage-dependent gating – one in which the voltage sensors move at the protein-lipid interface in response to a balance between hydrophobic and electrostatic forces. Rees and colleagues at the California Institute of Technology determined the structure of a voltage regulated mechanosensitive channel called MscS, and although it is unrelated to traditional voltage-dependent channels, it too contains hydrophobic helix-turn-helix elements with arginine residues apparently against the lipid membrane (Bass *et al.,* 2002). MscS and KvAP are fascinating membrane protein structures. They do not fit into the standard category of membrane proteins with rigid hydrophobic walls against the lipid membrane core. I find such proteins intriguing.

We are only just beginning to understand the structural principles of ion channel gating and regulation. Electrophysiological studies have uncovered a multitude of connections between cellular biochemical pathways and ion channel function (Hille, 2001). New protein structures are now beginning to do the same. Beta subunits of certain eukaryotic voltage-dependent channels are structurally related to oxido-reductase enzymes (Gulbis *et al.*, 1999; Gulbis *et al.*, 2000). PAS domains on other K^+ channels belong to a family of sensory molecules (Morais Cabral *et al.*, 1998), and a specialized structure on G protein-gated channels forms a binding site for regulatory G protein subunits (Nishida and MacKinnon, 2002). The interconnectedness of ion channel function with many aspects of cell function is beginning to reveal itself as complex and fascinating.

CONCLUDING REMARKS

I think the most exciting time in ion channel studies is just beginning. So many of the important questions are waiting to be answered and we have the tools in hand to answer them. I am very optimistic about the future, and for the great possibilities awaiting young scientists who are now setting out to study ion channels and other membrane proteins. I consider myself very fortunate to have contributed to some small part of the knowledge we have today. Of course, my contributions would never have been possible without the efforts and enthusiasm of the young scientists who have come from around the world to study ion channels with me (figure 16). I also owe thanks to the Rockefeller University, the Howard Hughes Medical Institute, and the National Institutes of Health for supporting my scientific research.

MACKINNON LABORATORY: 1989–2003

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Zhe Lu	Michael Root	John Lewis	Maria Garcia
Adrian Gross	Patricia Hidalgo	Alice Lee MacKinnon	Gerhard Wagner
Kenton Swartz	Sanjay Aggarwal	Sabine Mann	Andrzej Krezel
Chul-Seung Park	James Morrell	Richard Pfuetzner	Brian Chait
Rama Ranganathan	Alexander Pico	Anling Kuo	Steve Cohen
Chinfei Chen	Vanessa Ruta	Minhui Long	Martine Cadene
Declan Doyle	Ian Berke	Amelia Kaufman	Benoit Roux
John Imredy		Ernest Campbell	Tom Muir
João Morais Cabral		Jiayun Chen	
Youxing Jiang			
Jacqueline Gulbis			
Raimund Dutzler			
Francis Valiyaveetil			
Xiao-Dan Pfenninger-Li			
Ming Zhou			
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Yufeng Zhou			
Sebastien Poget			
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Uta-Maria Ohndorf			
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Qiu-Xing Jiang			
Seok-Yong Lee			
Stephen Long			

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Figure 16. MacKinnon laboratory from 1989 to 2003.

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