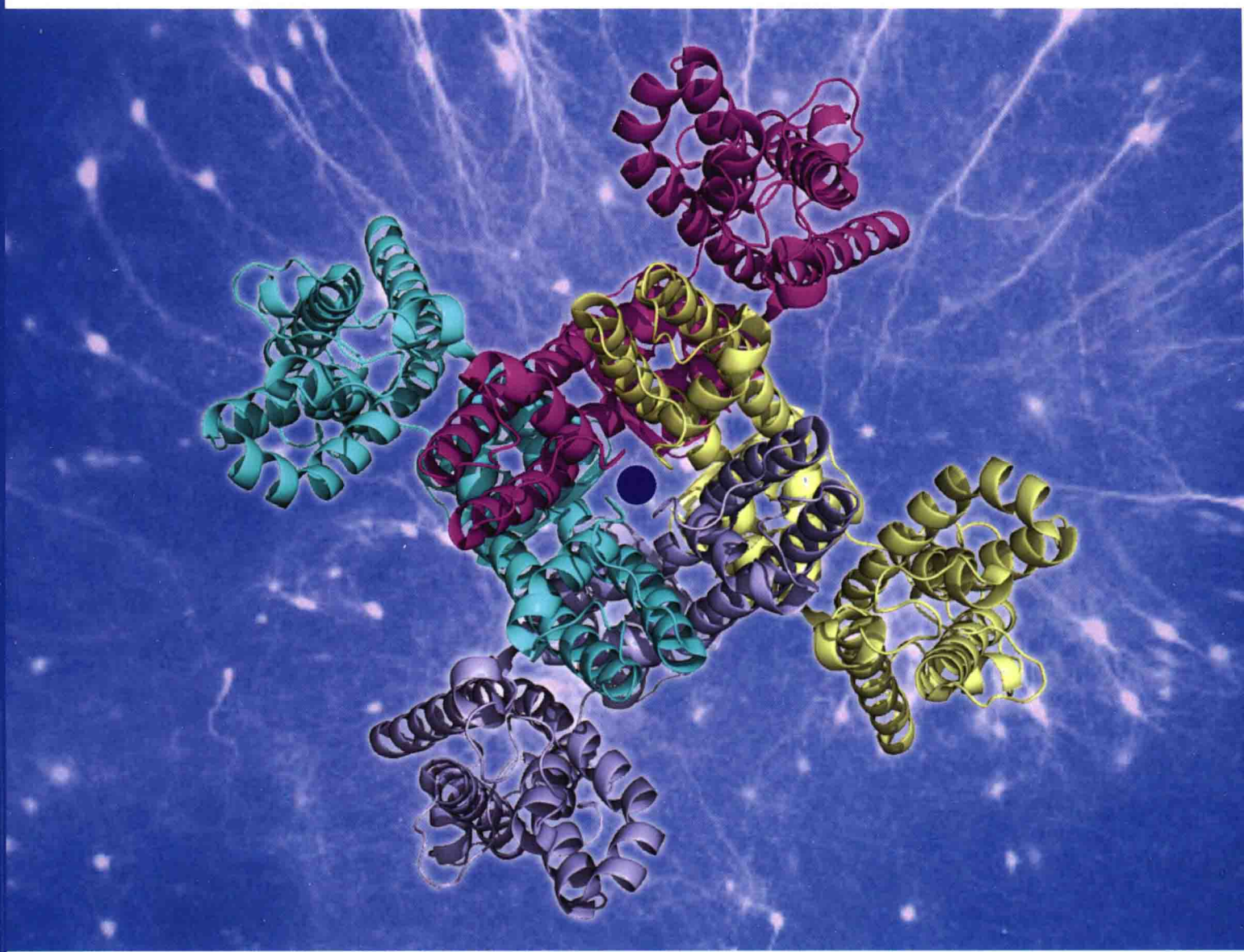
A LABORATORY MANUAL

Ion Channels



EDITED BY Paul J. Kammermeier, Ian Duguid, and Stephan Brenowitz

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ION CHANNELS
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Front cover image: (*Foreground*) A voltage-dependent potassium channel as viewed from outside of the cell membrane (PDB ID: 2R9R). Each subunit of the tetramer is uniquely colored. A potassium ion is shown as a blue sphere in the channel pore. The voltage-sensing domains are outside of the pore-forming domains. The structures and functions of voltage-gated potassium channels are reviewed in Chapter 2. Image courtesy of Dorothy M. Kim and Crina M. Nimigean. (*Background*) Flourescence image from a hippocampal slice culture. A protocol for preparing slice cultures from rodent hippocampus is included in Chapter 12. Image courtesy of Thomas G. Oertner.

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CHAPTER 1

Ion Channels: History, Diversity, and Impact

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From patch-clamp techniques to recombinant DNA technologies, three-dimensional protein modeling, and optogenetics, diverse and sophisticated methods have been used to study ion channels and how they determine the electrical properties of cells.

THE HISTORY OF ION CHANNELS

The modern era of studying electrical properties of excitable cells began in earnest in the 1930s. It was then that our understanding of how cells produce electrical potentials began to emerge and with it, an understanding of how bioelectric changes could be utilized to perform important cellular functions such as conveying information. As is often the case, technological advances helped drive scientific understanding. By the 1940s, Kenneth Cole and George Mormont had begun to develop the voltage clamp technique, wherein the membrane potential of a (large) cell could be measured and controlled, leading to the earliest descriptions of the electrical properties of membranes and the conductances that underlie neuronal action potentials. Soon after, Alan Hodgkin and Andrew Huxley refined the technique to discover that the action potential was not simply a relaxation of the membrane potential to zero, as had been previously thought, but constituted an overshoot of the membrane potential to positive potentials. Further, they discovered that the depolarizing phase of the action potential was due to sodium flux into the cell, while the repolarization back to the resting membrane potential was due to potassium efflux. It was for these findings that they, along with Sir John Eccles, received the 1963 Nobel Prize in Physiology or Medicine.

Thus began a long exploration of the mechanisms underlying the electrical properties of cells. During this time, competing ideas emerged to explain the discoveries of Hodgkin and Huxley. The first was that the plasma membrane itself changed confirmation to become selectively permeable to sodium, then to potassium. The alternative hypothesis, championed notably by Bertil Hille and Clay Armstrong during the 1960s, was that selective pores were formed by proteins in the membrane to facilitate the passage of ions into and into and out of the cell (T Begenesich, pers. comm.). Further, because of the observation that compounds like tetrodotoxin (TTX) and tetraethylammonium (TEA) could selectively inhibit sodium and potassium conductances, they reasoned that separate proteins most likely underlie these conductances. This was finally confirmed with the biochemical purification

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of the TTX binding protein (the sodium channel) from electroplax membranes by Agnew and colleagues in 1978.

Along the way, new conductances were discovered, including voltage- and ligand-gated conductances, but confirmation of the idea of voltage and ligand-gated pores formed by distinct proteins would require another technological advance, and this came with the development of the patch clamp technique by Bert Sakmann, Erwin Neher, and colleagues, in which they demonstrated that a very high resistance (GΩ) seal could be formed between a glass micropipette and the plasma membrane of a cell. This allowed the voltage clamp technique to be applied to much smaller cells than had previously been possible. Further, the technique could be applied to small patches of membrane, in some cases containing a single channel protein. Sakmann and Neher received the Nobel Prize in Physiology or Medicine in 1991 for the development of this method, which led to an explosion of discoveries in the areas of pharmacology, physiology, and of course ion channel biophysics. Recording of single ion channel currents using the patch-clamp technique remains the only method in biology in which the function of native individual protein molecules can be monitored in real time.

In the ensuing period, continued improvements and innovations have been made to the patch-clamp technique enabling more discoveries in areas previously thought intractable. These include iterative improvements to amplifier designs allowing more accurate and faster voltage clamp, and a multitude of innovations that have enabled patch-clamp recordings from single neurons in vitro and in vivo, patching of organelles (e.g., nuclear and mitochondrial membranes) and subcellular regions of cells (e.g., synaptic terminals, dendrites, cilia). These innovations have transformed our understanding of the role of ion channels, transporters, and pumps in both excitable and nonexcitable cells providing us with the ability, at least in mammalian experimental animals, to describe almost all cellular conductances at the molecular level.

THE ION CHANNEL SUPERFAMILY

The molecular level description of ion channel function was driven in part by the development of electrophysiological techniques but also by the parallel development of "recombinant DNA technologies" in the late 1970s, whereby individual genes could be reliably isolated, sequenced, purified, and cloned. The rapid development of gene sequencing methods, when combined with electrophysiology, created a way to unravel the true extent of mammalian ion channel diversity and to classify ion channels based upon sequence homology, gating properties, and phylogeny. The predicted amino acid sequences uncovered remarkable sequence homologies across groups of ion channels allowing biophysicists to classify "superfamilies" of homologous channel proteins that presumably evolved from common ancestral channels. Although recombinant DNA technologies were being developed in the late 1970s, it was not until the early 1980s that Masaharu Noda and colleagues first managed to clone, sequence, and describe the primary structure of the α-subunit precursor of the nicotinic acetylcholine receptor (nAChR, 1982) and voltage-gated sodium channel (1984) from the electric ray (*Torpedo californica*) and eel (*Electrophorus electricus*), respectively (Noda et al. 1982, 1984). This technical and conceptual enlightenment led to an explosion of interest in unraveling ion channel molecular diversity and to the classification of the two main ion channel superfamilies, voltage- and ligand-gated ion channels.

Voltage-gated ion channels, so called because of the requirement for a change in membrane potential to initiate "gating" or opening of the channel, were found to be tetrameric channels built from four homologous modules comprising a voltage sensor domain and pore-forming domain. Prominent among these are sodium, potassium, and calcium channels that underpin the action potential and calcium signaling cascades present in almost all electrically excitable cells. The biophysical properties and methods with which to isolate voltage-dependent conductances are described in detail in other articles in this collection. In addition to the more prototypic voltage-gated ion channels, several other phylogenetically related ion channels, such as cyclic nucleotide-gated (CNG)

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and transient receptor potential (TRP) channels, were identified due to their weak voltage dependence and retention of a voltage-sensing domain. In parallel, molecular identification and classification of ligand-gated ion channels, so called because of their requirement for extracellular ligand-binding to initiate a conformational change and opening of the ion channel pore, highlighted the presence of several families of ion channels gated by extracellular ligands. Prominent among these are ionotropic glutamate receptors (i.e., AMPA, Kainate, NMDA), cysteine-loop channels (i.e., Ach, GABA, glycine, 5-HT), ATP-gated channels (i.e., P2X) and phosphatidylinositol 4,5-bisphosphate (PIP_2)-gated channels. As the molecular identification of membrane channels gained pace through the 1980s and 1990s, the presence of additional, smaller families of ion channels such as calcium- or light-activated, cyclic nucleotide-gated, and mechanosensitive ion channels added to the increasingly diverse range of membrane proteins expressed by electrically excitable cells. The challenge that faced scientists of the time was to be able to unravel the complex biophysical mechanisms that link ion channel structure to physiological function.

An important feature of developing recombinant DNA technologies became the ability to apply site-directed mutagenesis to directly manipulate the amino acid sequence of a protein to establish a link between structure and function. The application of site-directed mutagenesis led to a number of groundbreaking discoveries including the mechanism of inactivation of Shaker potassium channels (Hoshi et al. 1991), identification of the region of the sodium channel forming the inactivation gate (Stühmer et al. 1989), pore-forming region of the potassium channel (Yellen et al. 1991) and an early description of the conformational change leading to "voltage sensing" in the Shaker potassium channel (Papazian et al. 1995; Larsson et al. 1996; Smith-Maxwell et al. 1998). This powerful genetic manipulation technique combined with advanced single cell electrophysiology heralded a new era for ion channel biophysics.

In parallel to the rapid expansion of recombinant DNA technologies, three-dimensional protein modeling and X-ray crystallography became an invaluable tool in the quest to understand how the amino acid sequence and predicted 3D secondary and tertiary protein structure related to biophysical characteristics of the ion channel. A paradigm shift in our understating of ion channel structure and function came in the early 1990s where Roderick MacKinnon became the first person to crystallize and analyze a potassium channel from the bacterium *Streptomyces lividans* (Doyle et al. 1998; MacKinnon et al. 1998), for which he later received the Nobel Prize in Chemistry in 2003. This powerful approach allowed scientists to visualize the structure of membrane bound proteins at an unprecedented level of resolution, forming 3D models of how sequences of amino acids interact to form a functional ion channel. This novel approach, when combined with emerging recombinant DNA technologies and single cell electrophysiology, generated a new vista of ion channel biophysics that has shaped the way we think about ion channel structure–function relationships to the present day.

RECOMBINANT CHANNEL TECHNOLOGY DRIVES MODERN NEUROBIOLOGY

Knowledge of the protein sequence, molecular structure, and gating behavior of ion channels has driven the development of an array of new methods directed toward understanding their biological function at the cellular, circuit, and behavioral levels. Many of the new approaches to understanding ion channel function rely on the use of light to achieve exquisite spatiotemporal control of channel gating when compared with pharmacological methods. Optical techniques for activating ion channels can be classified into several general approaches: (1) uncaging, the use of light to activate a chemically inert form of an ion channel ligand; (2) photoactivation, the direct activation of a photosensitive conductance such as channelrhodopsin; and (3) photoswitching, the use of light to isomerize a tethered ligand or channel blocker. Progress in each of these areas has resulted from both improvements in optical substrates resulting from molecular engineering and developments in microscopy, illumination, and imaging techniques.

An example of an early method for experimentally controlling ion channel activity is optical "uncaging" of glutamate to activate ionotropic glutamate receptors (Ellis-Davies 2007; Delaney and Shahrezaei 2013). The technique of uncaging requires synthesis of a chemically inert ("caged") form of a neurotransmitter, which upon exposure to light of the appropriate wavelengths (often in the UV portion of the spectrum) releases the biologically active form of the transmitter. Photolytic conversion of the caged to active form of the neurotransmitter can be achieved with brief light pulses (on the order of milliseconds) allowing the ligand to rapidly diffuse and activate its target receptor. Even greater spatial resolution can be achieved using two-photon activation of caged glutamate, which releases glutamate in a diffraction limited point ($\sim$1 μm^3) and can activate glutamate receptors on individually targeted dendritic spines in a manner similar to vesicular release of glutamate (Carter and Sabatini 2004; Svoboda and Yasuda 2006). Glutamate uncaging can be applied to many questions such as determining whether functional glutamate receptors are present on specific target neurons as well as their subcellular distribution, and characterizing the gating properties and kinetics of individual receptors. In addition to glutamate, many other neurotransmitters have been synthesized in a chemically caged form and are commercially available, such as GABA, serotonin, dopamine, and noradrenaline to name but a few.

Another method that has been highly influential in recent years is the optical activation of a cation conductance through activation of the algal photoreceptor channelrhodopsin (ChR2). When this light-gated ion channel is expressed in neurons, its activation produces a depolarizing current that can evoke action potentials. Numerous efforts have been made to increase the conductance and improve the kinetics of ChR2. Since the initial cloning of this receptor (Nagel et al. 2003) it has been widely adopted as a powerful tool for cell type–specific activation. This allows far greater resolution for mapping neural circuits (Petreanu et al. 2007) and the locations of synaptic inputs onto specific target neurons (Petreanu et al. 2009) when compared with electrical stimulation methods. The ability to suppress neuronal activity is also important for determining the roles of specific neurons in circuit function or disease. This has been achieved by optogenetic means by two main classes of protein, an inward chloride pump (halorhodopsin) and an outward proton pump (archaerhodopsin) (Chow et al. 2012).

Although optogenetic photoactivation has been enormously successful in determining circuit-level function, little information can be obtained when using this approach to define the roles of specific ion channels in cellular behavior. For this reason, development of photoswitchable tethered ligands has been a powerful and flexible tool for controlling the activation of specific types of ion channels with spatial, temporal, and molecular specificity (Szobota et al. 2007; Kramer et al. 2013). Several classes of ion channels have been rendered light-sensitive by attaching a channel blocker, such as a quaternary ammonium ion, to a linker such as azobenzene that undergoes a wavelength-dependent conformational change. This linker binds to a cysteine residue inserted near the pore of the Shaker potassium channel. At rest, or in the dark, this azobenzene group remains in the *trans* conformation and the pore is blocked by ammonium. Exposure to 380 nm light induces a change to the shorter *cis* conformation, and the ammonium ion is moved away from the pore, restoring conduction. This optically induced conformational change occurs on a submicrosecond time scale and can be reversed over many cycles. The ability to use light to control activation of ion channels allows far greater spatial and temporal specificity than conventional pharmacological methods.

Application of these widely diverse and sophisticated techniques has arisen to a large extent from the study of ion channels. A direct link can be traced from methods like optogenetics and its emerging dominant role in the broader fields of neuroscience and other arenas in biology, back to the tools initially developed to study channels and to discoveries of channels themselves. Further, analysis of single protein conformational changes in real time obtained with fluorescence techniques such as Förster resonance energy transfer (FRET) may be informed by the development of methods used to study single ion channels. In many ways, all of these techniques owe some debt to those original studies by Marmont, Cole, Hodgkin, and Huxley, and to the many scientists who followed them.

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CHAPTER 2

Voltage-Gated Potassium Channels: A Structural Examination of Selectivity and Gating

Dorothy M. Kim and Crina M. Nimigean

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Voltage-gated potassium channels play a fundamental role in the generation and propagation of the action potential. The discovery of these channels began with predictions made by early pioneers, and has culminated in their extensive functional and structural characterization by electrophysiological, spectroscopic, and crystallographic studies. With the aid of a variety of crystal structures of these channels, a highly detailed picture emerges of how the voltage-sensing domain reports changes in the membrane electric field and couples this to conformational changes in the activation gate. In addition, high-resolution structural and functional studies of K^+ channel pores, such as KcsA and MthK, offer a comprehensive picture on how selectivity is achieved in K^+ channels. Here, we illustrate the remarkable features of voltage-gated potassium channels and explain the mechanisms used by these machines with experimental data.

INTRODUCTION TO K^+ CHANNELS

Electrical signaling is produced by the interplay of a diverse array of ion channels, which are elegantly orchestrated to respond to a variety of stimuli and propagate this response in an efficient manner. Channel opening and closing is exquisitely balanced among different types of channels to correctly and precisely maintain homeostasis at resting potential and contribute to explosive electrical activity during the action potential. Potassium channels play a role in repolarization of the membrane, which follows membrane depolarization by sodium, and in some cases calcium, channels during the action potential; this is necessary for returning the membrane to a negative resting potential to terminate the action potential signal. The balance required for this interplay is achieved through the extremely high ionic selectivity, high rate of flux, and sophisticated gating mechanisms of potassium channels.

Potassium channels are found in all living organisms. These highly selective channels are conserved across the kingdoms and are present in all cell types including neurons, muscle cells, and other tissues. They have evolved to play different roles in different cells, yet they have retained key features that are conserved throughout the potassium channel family. One universal trait is their high selectivity; some potassium channels show up to 1000-fold preference for K^+ ions, over smaller ions such as Na^+ and Li^+ (Yellen 1984; Neyton and Miller 1988a; Hille 2001; LeMasurier et al. 2001). This high degree of selectivity is necessary for maintaining the resting membrane potential, with high K^+ inside and high Na^+ outside of the cell. This selectivity is achieved by the structure of a highly conserved amino acid sequence that forms a potassium ion selectivity filter within the pore.

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The membrane permeability of excitable cells to potassium ions was predicted by Julius Bernstein in 1902 (Bernstein 1902). He hypothesized that cells at rest were exclusively permeable to K^+ ions and that the cells were permeable to other ions only during periods of excitation, suggesting that there existed ion-selective components in the membrane. This hypothesis was borne out through the breakthrough studies by Hodgkin and Huxley performed on the squid giant axon in the 1940s and 1950s, which characterized the action potential in terms of the coordinated changes in the cell membrane permeability to Na^+ and K^+ ions and developed a model directly correlating these fluxes with excitation and electrical conduction (Goldman 1943; Hodgkin and Huxley 1945, 1946, 1947, 1952a,b,c,d,e,f; Hodgkin and Katz 1949; Hodgkin et al. 1952; Hodgkin and Keynes 1955). This was correctly hypothesized at the time to result from the orchestrated opening and closing of voltage-dependent ion-selective channels. Their experiments confirmed that the cell was predominantly selective for K^+ ions when it was at rest, resulting in a negative transmembrane potential (measured as the inside of the cell relative to the outside). These studies were key in highlighting the importance of both ion selectivity and regulated gating to generation of the action potential.

In addition to being highly selective, K^+ channels conduct ions at an extremely fast rate, close to the rate of diffusion (Hille 2001). How is this possible? Again, the clever architecture of the selectivity filter provides the answer to this paradox. Structural and functional studies of potassium channels have shown that the placement of ions in the selectivity filter leads to a "knock-on" mechanism (Fig. 1), which exploits the charge-charge repulsion of the K^+ ions that march in single file through the filter (Hodgkin and Keynes 1955; Doyle et al. 1998). Thus, the selectivity filter has evolved to efficiently conduct ions in a highly selective manner, allowing the fast rate of flux required to quickly repolarize the cell.

K^+ channels must respond rapidly on sensing changes in cellular environment. Ion flux is controlled by channel gating; that is, the ions cannot pass through the selectivity filter unless the channel is open, and the flow of ions must also be controlled by the ability of the channel to close. To do this rapidly, potassium channels have evolved gating mechanisms to couple the sensing of environmental cues with physical movements of domains within the channel that control ion flux. A major family of potassium channels is the voltage-gated potassium channels (Kv), which detect changes in transmembrane voltage and couple this detection to channel opening and closing.

Here, we will discuss the mechanisms by which K^+ channels achieve high selectivity while retaining a fast rate of flux, how the channel senses the change in transmembrane voltage, and how this detection of voltage is coupled to gating of the channel.

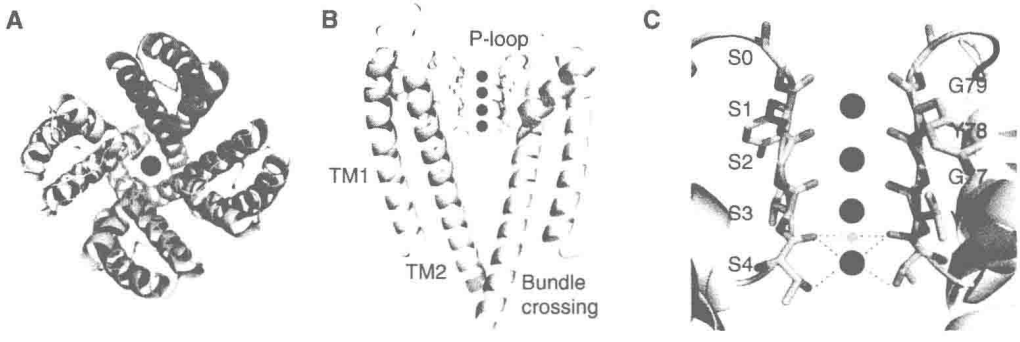

FIGURE 1. Structural details of KcsA. (*A*) KcsA tetramer as viewed from the top of the membrane (PDBID:1 4KC). Each subunit is uniquely colored. The central ion conduction pore is shown with a K^+ ion (blue sphere). (*B*) KcsA as viewed from the side with two opposing subunits removed for clarity. The P-loop contains the signature sequence TVGYG (shown as sticks), and forms the selectivity filter. Four potassium ions are shown in the selectivity filter. Below the selectivity filter is the aqueous cavity, formed by the TM2 helices that form the bundle crossing. (*C*) A detailed view of the selectivity filter of KcsA. Each binding site, S0–S4, is formed by the oxygen cages originating from backbone carbonyl and side-chain hydroxyls of the selectivity filter signature sequence TVGYG. Dashed lines depict coordination of the K^+ ion in S4 and the oxygens. Sodium (orange sphere) binds in the plane between sites S3 and S4. Dashed lines represent its coordination with carbonyl oxygens.