Intra-membrane Molecular Interactions of K$^+$ Channel Proteins: Application to Problems in Biodefense and Bioenergy

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Abstract

Ion channel proteins regulate complex patterns of cellular electrical activity and ionic signaling. Certain K$^+$ channels play an important role in immunological biodefense mechanisms of adaptive and innate immunity. Most ion channel proteins are oligomeric complexes with the conductive pore located at the central subunit interface. The long-term activity of many K$^+$ channel proteins is dependent on the concentration of extracellular K$^+$; however, the mechanism is unclear. Thus, this project focused on mechanisms underlying structural stability of tetrameric K$^+$ channels. Using KcsA of *Streptomyces lividans* as a model K$^+$ channel of known structure, the molecular basis of tetramer stability was investigated by: 1. Bioinformatic analysis of the tetramer interface. 2. Effect of two local anesthetics (lidocaine, tetracaine) on tetramer stability. 3. Molecular simulation of drug docking to the ion conduction pore. The results provide new insights regarding the structural stability of K$^+$ channels and its possible role in cell physiology.

ACKNOWLEDGMENTS

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Nomenclature

CaM	calmodulin
ChR	channelrhodopsin
ER	endoplasmic reticulum
IP_3	inositol-trisphosphate
IP_3R	IP_3-receptor – Ca^{2+} release channel
KcsA	K^+ channel protein of *Streptomyces lividans*
LA	local anesthetic
LPS	lipopolysaccharide
NO	nitric oxide
PAMP	pathogen-associated molecular pattern
PIP_2	phosphatidylinositol 4, 5,-bisphosphate
PLC	phospholipase C
SOCE	store-operated Ca^{2+} entry
TBA^+	tetrabutylammonium
VGL	voltage-gated like channel family

1. INTRODUCTION

1.1 Introduction to Ion Channel Proteins and Relevance to Biodefense and Bioenergy

A thin ~4 nm structure called the plasma membrane is the boundary between the elementary unit of Life, the Cell, and the non-living matter of the cell's immediate environment. As such, the membrane is both a protective barrier and a communication interface. It determines which ions and small molecules enter and leave the cell and the rate at which each of the recognized substrates move across the barrier. Trans-membrane fluxes of inorganic ions and transported substrates maintain osmotic stability of the cell within physiological limits while the membrane maintains flexibility to alter its shape according to function (e.g., endocytosis, exocytosis, migration). The membrane also senses diverse external stimuli (e.g., temperature, touch, light), molecular messengers (e.g., hormones, cytokines), dangerous substances (e.g., toxins), and potentially harmful biological intruders (e.g., bacterial and viral pathogens). It functions as a physical platform for a complex system of signaling mechanisms that mediate chemical communication between the outer community of the local cell population and the inner network of the cell's own genes, cytoplasmic organelles, and biochemical machinery. All of these sophisticated functions of cell membranes are mediated by many diverse nanomachines in the form of membrane proteins— enzymes, receptors, transporters, pumps, and ion channels. A recent analysis determined that 26% (5,539 genes) of 21,416 annotated genes in the current database of the human genome are integral membrane proteins (1).

Native cell membranes consist of a phospholipid bilayer with numerous integral (i.e., embedded) membrane proteins and peripheral proteins attached at the membrane surface. Charged or polar molecules do not readily dissolve in the interior hydrocarbon phase of the membrane composed of alkyl lipid chains. Thus, the hydrophobic inner core of the bilayer effectively prevents trans-membrane permeation of ions and hydrophilic molecules such as water, sugars, and amino acids. Ion channels are a unique class of membrane proteins that subserve fast electro-diffusive movement of inorganic ions (primarily Na^+, K^+, Ca^{2+}, H^+, and Cl^-) across cell membranes (2). In structural terms, a channel protein forms a gated pore across the membrane that allows certain ions and water molecules to rapidly diffuse across the membrane when the pore is open. The pore sizes of such channel proteins are often close to the atomic diameter of the permeant ions. Thus, many channels select ions by a sieving mechanism that involves in the single-file movement of ions and water molecules through the pore. Fast movement of ions through channel proteins corresponds to ionic currents that underlie electrical signaling behavior of neurons and muscle cells. Since electrical signaling is the basis of sensory information acquisition and fast intelligent response behavior of living organisms, biological ion channels have been compared to the transistor elements of computer microprocessors (3).

Bioelectric signaling is based on changes in membrane potential (i.e., the voltage difference across the membrane) within a range of about -100 to +50 mV. (The sign of the voltage is referenced to extracellular ground.) Such changes in membrane potential correspond to the temporal pattern of opening of particular ion channels which drives the membrane toward

a voltage corresponding to the Nernst equilibrium potential, E_X, for different ions (X) as given by the following form of the Nernst Equation:

$$E_X = \frac{RT}{zF} ln \frac{[X]_{out}}{[X]_{in}}$$

$$Eq. 1$$

where R is the gas constant, T is absolute temperature, z is the charge valence of the ion, F is the Faraday constant, and $[X]_{out}$ and $[X]_{in}$ are the concentrations of ion X at the external and internal sides of the membrane, respectively. For example, tightly regulated concentration gradients of inorganic ions across mammalian cells correspond to approximate Nernst equilibrium potentials of: E_K = -90 mV, E_{Na} = +60 mV, E_{Ca} = +120 mV, and E_{Cl} = -50 mV.

The so-called resting potential of a typical cell (i.e., when the cell is not being excited by a stimulus) has a value of about -80 mV due to the dominant membrane K^+ permeability mediated by certain K^+-selective channels open at rest. Cellular excitation leading to initiation of nerve action potentials, muscle contraction, or neurotransmitter release is triggered by the opening of Na^+- or Ca^{2+}-selective channels which drives V_m to a positive value. Such a positive-going change in V_m is termed depolarization. Conversely, cessation or inhibition of excitation is due to the opening of K^+ or Cl^- channels which cause a negative-going change in V_m is called hyperpolarization. Inasmuch as channel-mediated electrical signaling is well-known to underlie inter-cellular communication of the nervous system (2), ion channels have also been found to play a key role in many other aspects of cellular physiology, including those related to biodefense and bioenergy.

For example, ion channels have been found to play a critical role in the process of antigen activation of T and B lymphocytes in the adaptive immune response (4, 5). Briefly, antigen binding to the T-cell receptor is coupled to activation of PLC (phospholipase C) via phosphorylation by tyrosine kinases such as cSrc and Syk. The hydrolysis of PIP2 (phosphatidylinositol 4,5-bisphosphate) by PLC to yield inositol-trisphosphate (IP_3) and diacylglycerol. IP_3 activates release of Ca^{2+} from endoplasmic recticulum (ER) stores via the IP_3-receptor/ Ca^{2+} release channel (IP_3R). Sustained elevation of intracellular Ca^{2+} necessary for transcriptional activation of cytokine genes is achieved by store-operated Ca^{2+}-entry (SOCE) across the plasma membrane. The electrophysiological hallmark of SOCE is an inward Ca^{2+} current known as I_{CRAC} (Ca^{2+}-release activated current) (6, 7). Groundbreaking work recently clarified the molecular elements of SOCE and I_{CRAC} (8-12). Depletion of ER Ca^{2+} is sensed by Ca^{2+} dissociation from the N-terminal EF-hand domain of the STIM ER transmembrane protein. Oligomers of activated STIM directly interact with the intracellular portion of ORAI protein dimers in the plasma membrane to form an active tetrameric ORAI Ca^{2+} channel. Ca^{2+} entry through the ORAI channel is coupled to Ca^{2+}/Calmodulin activation of calcineurin, a protein phosphatase that dephosphorylates NFAT transcription factor, allowing its nuclear localization and activation of cytokine genes. These complex cellular mechanisms illustrate the essential role of Ca^{2+}-selective channels in the process of lymphocyte activation that controls the antibody response to harmful pathogens. However, an appreciation of subtler biophysical dependencies is needed to understand how this Ca^{2+} influx response is functionally dependent on certain K^+ channels.

Robust Ca^{2+} influx requires an electric countercurrent to balance charge movement across the membrane. It also requires a negative-inside membrane potential (V_m) as a driving force to support Ca^{2+} entry. In lymphocytes, two different K^+ channel proteins, Kv1.3 and KCa3.1, perform this function (4, 5). Kv1.3 is member of the voltage-activated K^+ channel family and KCa3.1 belongs to the SK/IK family of low conductance K^+ channels activated by Ca^{2+}/CaM. The essential role of K^+ channels in lymphocyte activation is revealed by the ability of various K^+ channel blocking drugs and toxins to suppress T-cell proliferation and cytokine production (4). Lymphocyte K^+ channels are now recognized as an important target for immunosuppressive drugs that may be useful in the treatment of human autoimmune diseases such as multiple sclerosis (13-17).

Although the detailed mechanisms are less well understood, ion channels are also intimately involved in another class of defensive mechanisms known as the innate immune response. This response refers to a variety of evolutionarily ancient responses that are activated upon exposure to pathogen-associated molecular patterns (PAMPs) that include various foreign substances, and endogenous "danger signal" molecules (18-21). Phagocytic leukocytes including macrophages, neutrophils, and dendritic cells are key effectors of innate immune processes that include inflammation, killing and removal of pathogens, and subsequent activation of the adaptive immune response (22). Macrophages play a pivotal role in inflammation, in a process that involves production of chemical mediators capable of cellular damage (20, 23).

For example, exposure of macrophages to lipopolysaccharides (LPS, endotoxin) of Gram negative bacteria (e.g. *E. coli, Salmonella*) triggers cellular responses associated with septic shock, a major cause of mortality in immune-compromised patients (24). Signal transduction mechanisms involving lipid signaling and ion channels are responsible for onset of impaired vasoconstriction in sepsis. Specifically, LPS activates a Toll-like receptor (TLR4) pathway that results in excessive production of numerous inflammatory mediators [e.g., TNF-α, IL-1β, arachidonic acid (AA), eicosanoids, nitric oxide synthase (iNOS), and nitric oxide (NO)] by macrophages and vascular endothelial cells (23-26). Endothelial NO activates BK_{Ca} channels (KCa1.1) in neighboring vascular smooth muscle cells resulting in runaway vasodilation and hypotensive shock (27-29). Evidence for the functional roles of ion channels in the innate immune responses of macrophages is too extensive to describe here in detail. As an illustration this complexity, the schematic diagram of Figure 1 depicts the biochemical network of electrochemical coupling of the macrophage TLR4 pathway to intracellular Ca^{2+} release, extracellular Ca^{2+} entry, and five different K^+ channels known to be expressed in macrophage cells.

Ion channels are also important in processes related to bioenergy production and biofuels. For example, a number of K^+ channel proteins are expressed in mitochondria where they are involved in regulation of oxidative energy metabolism (30). K^+ channels of green plants have been found to play an important role regulation of cell growth, osmotic balance, and environmental stress responses (31). Motile algae such as *Chlamydomonas* that produce triacylglycerol used for biodiesel production express a light-activated ion channel known as channelrhodopsin (ChR). ChR is involved in phototactic and photophobic sensory responses that allow the unicellular organism to migrate to optimal light conditions (32). Given that ion channel proteins typically function in the conversion of various forms of energy (e.g., chemical,

mechanical, heat, light) into an electrical signal, they are considered to be useful models for the biomimetic design of nanobiosensors and nanobioelectronic components of novel devices (33). In conclusion to this introductory overview, the science of ion channels is a source of numerous applications relevant to pathogen defense and bioenergy technology.

Figure 1.

Model of TLR4 Signaling in Response to Bacterial Endotoxin Illustrating the Functional Role of Ca^{2+}- and K^{+}-selective Ion Channels.

Macrophage Activation in Sepsis and Inflammatory Disease
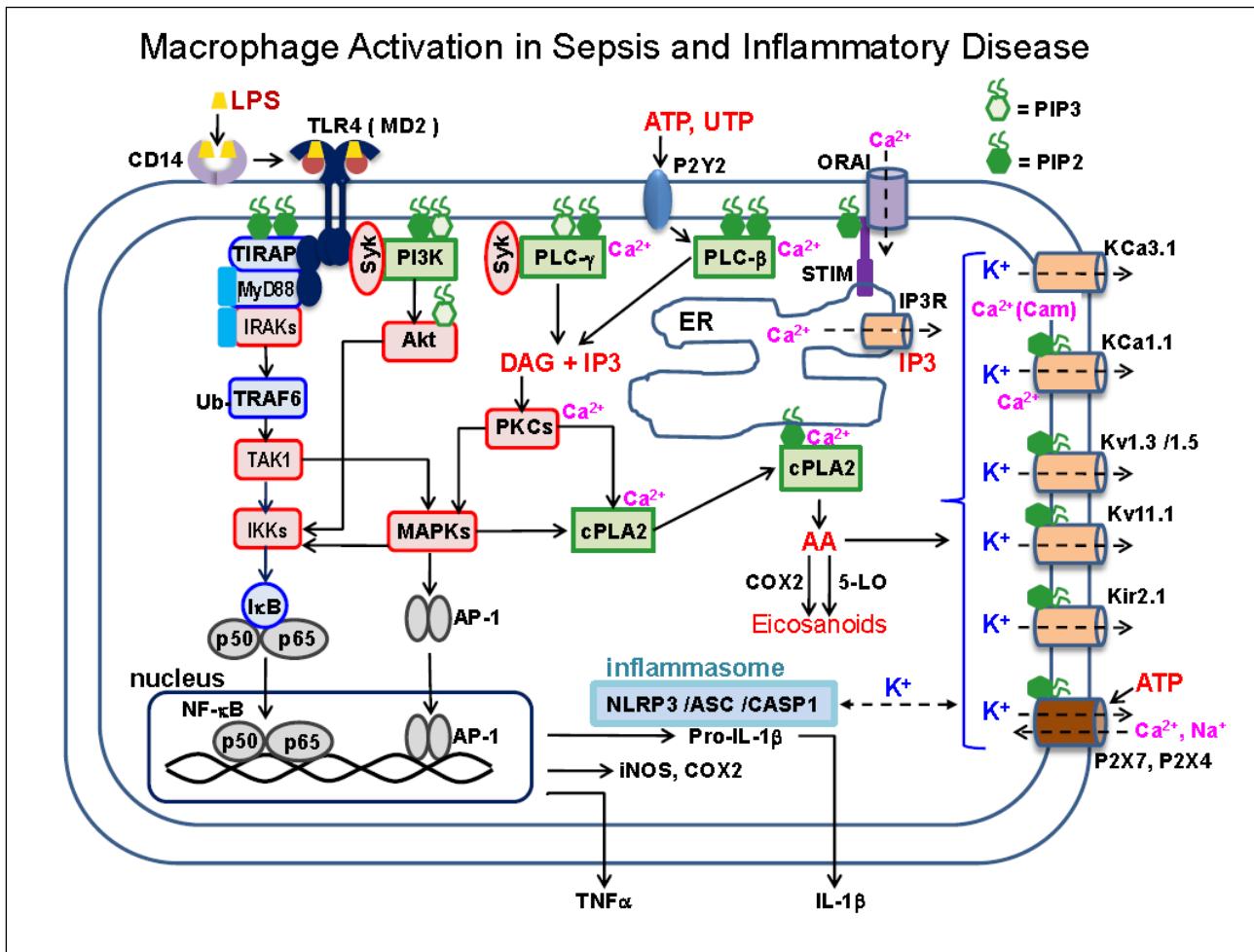
1.2 Structure and Function of Ion Channels: What Holds the Channel Together?

The common structural feature of all ion channel proteins is an ion-conductive pore— essentially a hole through the protein. The conduction pore acts like low resistance element in an electrical circuit, it provides an easy route for current to flow across the otherwise high-resistance cell membrane. The atomic dimensions and electrostatics of the channel pore determine the magnitude of the unitary conductance, g_{ch}, for different ions. g_{ch} is equivalent to the reciprocal of the electrical resistance (R) of the pore. The magnitude of g_{ch} is also a signature parameter that can be used to identify the channel by single-channel recording, a technique which resolves the open-closed current fluctuations of single channel molecules. Such current records obtained at different voltages can be used to analyze the current-voltage (I-V) behavior of the channel of interest and compute g_{ch} from the slope of the I-V relationship. The magnitude of g_{ch} for biological ion channels is typically within the range of 1-300 pS (1 pS = 10^{-12} Siemens; Siemens = $Ohms^{-1}$). Many channels exhibit a linear I-V curve for the open channel (at least over a certain low-voltage range) that is indicative of ohmic behavior according to Ohm's Law:

$$V = I\,R \ \ (or) \ \ g_{ch} = R^{-1} = I\,/\,V \ \ (or) \ \ I = g_{ch}V \hspace{3cm} Eq.\ 2.$$

Another signature feature of an ion channel is its "gating" behavior. Permanently open channels are generally detrimental to cellular physiology since they promote leakage of ions, dissipation of ionic gradients, and loss of function for electrical signaling. Consequently, most channels typically reside in a closed state most of the time and only open under well-defined conditions to effect certain functions. "Gating" thus refers to mechanisms involved in the opening and closing of the channel pore. As the term suggests, such opening and closing events are "gated" by conformational changes coupled to structural changes at the narrowest region of the ion conduction pathway. Ion channels utilize many different gating mechanisms. For example, various types of biological ion channels are gated by changes in voltage, binding of diverse ligands (e.g., Ca^{2+}, H^+, cyclic AMP, cyclic GMP, ATP, acetylcholine, glutamate, serotonin, glycine, γ-aminobutyric acid, lipids), mechanical deformation of the membrane, changes in temperature, and absorption of light.

Given the remarkably diverse conductance and gating behavior of ion channels, considerable effort has been devoted to analyzing the structural basis of these mechanisms. This effort has been greatly advanced in recent years by development of X-ray crystallographic methods to determine the structure of channel proteins at atomic resolution. Such studies have provided a wealth of information on the 3-dimensional structure of channel proteins.

Structural analysis has revealed that the ion conduction pore of most channel proteins is formed at the center of a radially symmetric or pseudo-symmetric rosette of subunits. This principle is illustrated in Figure 2 which classifies different families of ion channel proteins according to their oligomeric structure. The donut-shaped pie diagrams of Figure 1 are meant to illustrate the schematic quaternary structure of the listed ion channels as viewed from the top or bottom plane of the cell membrane. Many ion channels are formed as a homo-oligomeric complex of a single integral membrane protein subunit. For example the channel-forming subunit of certain K^+ channels assembles as a homo-tetramer with a four-fold axis of rotational

symmetry located at the center of the pore oriented normal to the plane of the membrane. In contrast, certain ligand-gated channels such as the nicotinic acetylcholine receptor (AChR) are formed as a hetero-pentamer of four non-identical but homologous subunits with the composition $\alpha_2\beta\gamma\delta$. Other channels such as voltage-gated Na^+ and Ca^{2+} channels (NaV, CaV) are formed from a single large polypeptide that contains four repetitive homologous domains, but which folds into a pseudo-tetrameric structure similar to tetrameric K^+ channels. Thus, for the different channels listed in Figure 2, all of which are present in the human genome, the ion conduction pore is located at the central interface of either a trimer, tetramer, pentamer, or hexamer of subunits arranged in a circular configuration (2).

Since structural integrity of the pore is essential to the function of a channel protein, molecular mechanisms involved in inter-subunit recognition and structural stability of the quaternary complex are expected to be of major physiological significance. For example, many mutations of ion channel genes that underlie human genetic diseases affect folding and structural stability of the channel protein. These mutational defects often result in misfolding, defective assembly, and poor expression of functional channels in the plasma membrane. Low expression of certain ion channels results in pathological conditions such as cystic fibrosis or inherited propensity for cardiac arrhythmia (34). Despite their significance, molecular interactions that determine the structural stability of oligomeric subunit complexes of ion channels are poorly understood. Thus, the objective of this EC-LDRD project was to develop new approaches to investigate the quaternary structure stability of tetrameric K^+ channel proteins. The overarching question posed in this research is: What holds the K^+ channel tetramer together?

Figure 2.

Oligomeric Structure of Most Channel Proteins: Identical or Homologous Subunits Surrounding the Central Pore Axis

1.3 KcsA as a Model K$^+$ Channel Protein

Tetrameric K$^+$ channels were chosen as the focus of study in this project based on their functional significance as cellular regulators of membrane potential, the availability of high-resolution structures, and ease of biochemical manipulation. In particular, the KscA gene from *Streptomyces lividans* was selected for experimentation. Historically, KcsA was the first K$^+$ channel protein to be successfully studied by X-ray crystallography (35). Correspondingly, there is a large body of mechanistic information on this protein. Figure 3 shows the 3D structure of the closed conformation of the membrane-spanning pore-domain of KcsA using a ribbon backbone representation to illustrate α-helical regions of the protein. The left panel shows a top view of the KcsA tetramer looking down on the extracellular surface of the membrane; the right panel is a side view.

The top view of the KcsA channel in Figure 3 illustrates the tetrameric radial symmetry of 4 identical monomer subunits oriented by consecutive 90° rotations about the central axis.   The monomer pore domain consists of three distinct segments of α-helix as approximately indicated by the consecutive blue, gray and red segments starting from the N-terminus. The N-terminal blue helix (M1) and C-terminal red helix (M2) both span the whole width of the membrane. The N-terminal or outer helix (M1) is located on the exterior of the tetramer and mainly faces the lipid bilayer while the C-terminal or inner helix (M2) forms the cytoplasmic half of the ion conduction pore. The M2 inner helix is also tilted at an angle with respect to the membrane such that the four C-terminal ends of this helix cross near the cytoplasmic face of the membrane to form the narrowest constriction of the pore.   Gating transitions from the closed to open state of the KcsA K$^+$ channel (see Figure 6) is pH-dependent with channel opening increasingly favored from pH 8 to pH 4. The outer and inner helices are connected by a short α-helical region called the pore helix (colored gray in Figure 3). From the N- to C- direction of the polypeptide, the pore helix begins after the external loop at the C-terminal end of the outer helix and enters the membrane at an angle tilted toward the center of the conduction pore. The pore helix traverses the outer half of the bilayer before turning sharply outward to form a strand of the selectivity filter consisting of conserved residues T$_{75}$VGYGD$_{80}$ (see Figures 4 & 14). The latter peptide segment that forms the selectivity filter emerges at the extracellular side of the channel to form part of the conical external vestibule of the pore and ends in a short external loop that connects to the M2 inner helix.

Figure 3.

X-ray Crystal Structure of the KcsA K$^+$ Channel Represented as a Ribbon Diagram of the Peptide Backbone
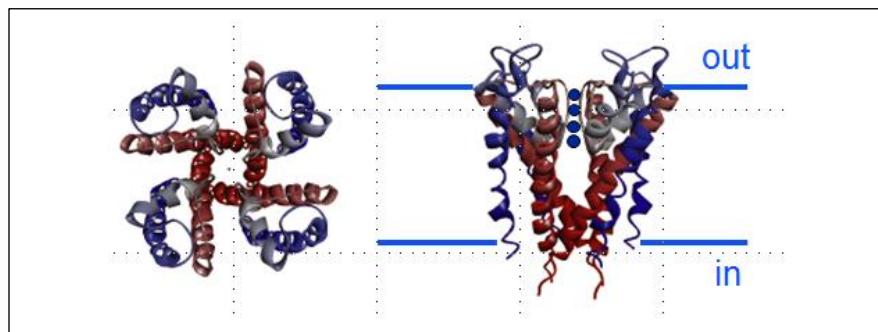
The selectivity filter of KcsA corresponds to four closely apposed peptide segments (36, 37), one from each monomer, in close proximity to four dehydrated K^+ ions (i.e., four blue spheres visible in the left part of Figure 3). The signature K^+ channel filter sequence, TVGYG, is highly conserved in evolution. In fact, it appears that the structure of the KcsA pore domain and its conserved filter sequence is a universal structure for K^+-selective channels in all living organisms— no other membrane channel structure that displays high selectivity for K^+ has ever been discovered.

The structure of the filter is shown at higher resolution in Figure 4 where two KcsA monomer segments, one in front and one in the rear, have been removed for clarity. The locations of four K^+ ions in the filer correspond to four binding sites for K^+ known as S1, S2, S3, and S4, shown as numbered from the outer to inner end of the filter . Each of these filter K^+ binding sites is formed as a cage of coordinating oxygen with four oxygen atoms lying in a plane above and below each of the four ion-binding sites. All of the coordinating oxygen atoms are carbonyl oxygen atoms (=O) of the peptide backbone except for the innermost plane of oxygens of the S4 site which consist of four hydroxyl (-OH) oxygen atoms of the four side chains of residues Thr75. Figure 4 also shows two addition K^+ ions located external to the narrow filter region that are stabilized by the negative electrostatic potential in this region. KscA crystal structures also often contain another hydrated K^+ ion located in the central region of the conduction pore that is known as the central cavity (36, 37). The central cavity can be seen as an empty space in the center of the channel as viewed in the left panel of Figure 3. This cavity is mainly lined by side chains of hydrophobic residues and has been shown to bind organic cations such as tetraethyammonium (TEA$^+$), a drug-like molecule which is known to block K^+ current through the channel (38-41).

Figure 4.

Structure of the Selectivity Filter Region of KcsA Including Residues Thr74 - Asp80
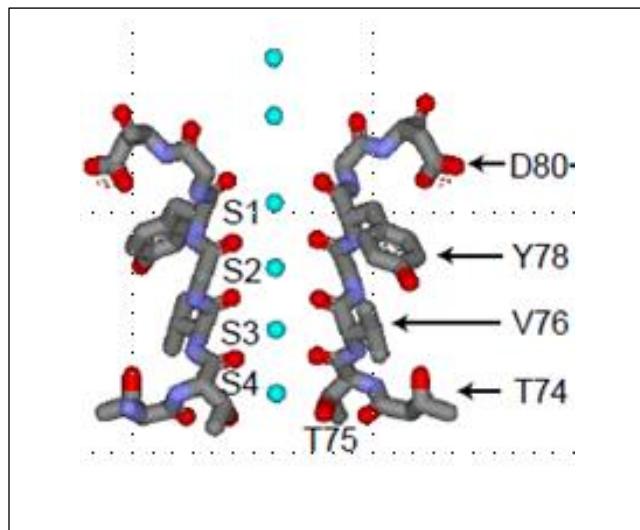
1.4 Hypothesis and Experimental Approach

The tetramer formed by four KcsA monomers is an extraordinarily stable oligomeric complex. For biochemical studies, recombinant KcsA protein is typically expressed in *E. coli*, solubilized and purified in a mild non-ionic detergent such as dodecylmaltoside. When the purified protein is analyzed by SDS-PAGE (sodium dodecylsulfate polyacrylamine gel electrophoresis), a technique that separates proteins according to molecular mass, KcsA migrates as an intact tetramer (42). Such behavior is rather unusual for oligomeric proteins which commonly dissociate into constituent subunits in the presence of SDS.

The crystal structure of the KcsA tetramer provides insight to the molecular origin of its extraordinary tetramer stability. The structure suggests that inter-subunit (monomer-monomer) contact regions of the protein and K^+ ions bound at the central interface of the selectivity filter may be especially important. Certain types of phospholipids such as phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) have also been found to enhance tetramer stability of KcsA relative to phosphatidylcholine (PC), suggesting that specific lipid-protein interactions and/or bulk mechanical properties of the bilayer may also contribute to tetramer stability (43-45). However, since detergent-solubilized KcsA maintains its tetrameric structure in the absence of added phospholipids, lipid interactions are not an absolute requirement.

We previously investigated the role of inorganic cations by purifying recombinant KcsA protein solubilized in dodecylmaltoside micelles and exchanging the purification buffer with 10 mM Hepes-Tris, pH 7.4, and 100 KCl or other salts of interest by consecutive rounds of centrifuge ultrafiltration (46, 47). The samples were assayed for thermal stability by exposing them to 10-minute incubation at various temperatures followed by determination of tetramer content on SDS-PAGE. The results showed that inorganic cations that readily permeate through K^+ channels (e.g., K^+, Rb^+) or cations that effectively block the channel (e.g., Cs^+, Ba^{2+}), effectively stabilize the KscA tetramer at temperatures tested up to 100 °C. In contrast, non-permeant cations (e.g., Na^+, Li^+, choline$^+$) shift the midpoint temperature of tetramer dissociation to a much lower range of ~60–80 °C. Ion-dependent stabilization of the KcsA tetramer is also a function of the concentration of inorganic cations present in the assay mixture. Titration of KcsA with increasing concentrations of K^+ or other stabilizing cations increased the fraction of observed tetramer content from 0 to 100% with a midpoint concentration of 1.5 mM K^+ for 50% tetramer (46).

Such cation titration experiments suggested that binding of K^+ and certain other inorganic cations that stably bind to the selectivity filter effectively protect against thermal dissociation of the tetramer. This hypothesis was validated by experiments that analyzed the effect of several mutations of Thr75, the KcsA residue that contributes four oxygen ligands as side chain –OH groups to the S4 binding site for K^+ (see Figure 4). The results confirmed that binding of K^+ or certain other inorganic cations to specific sites in the selectivity filter is a major determinant of K^+ channel tetramer stability (47).

Although the basic 3D structure of the pore domain of KcsA is shared by many related types of ion channels, the extraordinary thermal stability of the KcsA tetramer may not be

a universal feature of all such ion channels. For example the human genome contains ~137 channel genes with a pore domain homologous to KcsA. This large group of channel proteins has been termed the voltage-gated-like channel superfamily (VGL-channels) since it includes genes that code for voltage-gated (V-gated) cation channels selective for K^+, Na^+, Ca^{2+}, and non-selective cation channels (48). The VGL-superfamily contains 78 genes for K^+ channels, 10 genes for cyclic-nucleotide activated channels, 11 genes for V-gated Ca^{2+} channels, 10 genes for V-gated Na^+ channels, and ~28 genes for TRP non-selective cation channels that mediate a diverse array of sensory responses. Very few of these channel proteins have been studied biochemically or assayed for tetramer stability. Sequence alignment indicates that there is great divergence in the residues predicted to form the inter-subunit interfaces among the VGL gene family of tetrameric and pseudo-tetrameric channels. Thus it is likely that some of these channel proteins may exhibit weaker or stronger tetramer stability than KcsA. These considerations lead to the following **hypothesis: variations in tetramer stability of different types of VGL channels may serve as a physiological mechanism for regulating channel expression and bioelectrical activity.**

As an example relevant to this hypothesis, over 290 genetic mutations of the human cardiac hERG K^+ channel, also known as Kv11.1, are linked to Long-QT Syndrome 2 (LQT2), a condition characterized by susceptibility to cardiac arrhythmia, ventricular fibrillation, and sudden death (49). Most of the natural LQT2 mutations cause structural instability of the hERG channel protein and failure of the newly synthesized hERG to appropriately traffic from the cytoplasm and incorporate into the plasma membrane (49-51). Studies of hERG channel instability have also shown that low extracellular K^+ results in rapid conversion of the native channel to a non-conducting state followed by rapid decline in surface density of the channel protein (52). Several other types of K^+ channels have been observed to exhibit reversible or irreversible loss of function when exposed to low concentrations of K^+ on one or both sides of the membrane (53-59). These observations suggest that K^+-dependent tetramer stability may be a common characteristic of many different K^+ channel proteins.

To pursue this hypothesis systematically it would be useful to develop convenient quantitative methods to monitor the oligomeric status of any K^+ channel protein. Ideally such methods would be capable of monitoring the distribution of monomers, tetramers and other intermediate oligomeric states of K^+ channels in real time using a variety of experimental formats such as detergent micelles, model membranes (e.g., liposomes), and native cell membranes. Various fluorescence spectroscopic methods such as fluorescence resonance energy transfer (FRET), single-molecule tracking, excimer formation, and polarization techniques are attractive options for technical development of such assays since they are capable of monitoring proximity-dependent, protein-protein interactions with the use of appropriate probes and labeling techniques. It was hoped that such methods could be developed within the limited 2-year timespan of this project but delays in setting up the laboratory and other contingencies necessitated a different approach.

This involved a study of the binding interaction between organic cation drugs and K^+ that binds selectively to the filter region. As mentioned above, certain organic cations such as tetraethylammonium (TEA^+) are known to gain access to the central cavity of K^+ channels and block ion conduction through the pore (38-41). We hypothesized that repulsive electrostatic

interactions between an organic cation bound in the central cavity and K^+ ions bound to neighboring sites in the filter, such as the S4 site, could destabilize the tetramer by decreasing the K^+ occupancy of the filter. Additionally, it is possible that certain organic cations might interact with amino acid residues at the inter-subunit interfaces of the tetramer and perturb chemical interactions at these interfaces that also stabilize the tetrameric assembly.

To test this idea we chose two organic cation molecules, lidocaine and tetracaine, that belong to a well-known class of clinical drugs known as local anesthetics (LAs). Such LA drugs are known to interact with VGL channels by blocking ionic current (60, 61). Two of the most important clinical applications of LA drugs are for induction of local anesthesia in minor surgery and childbirth and for the use in the treatment of certain types of cardiac arrhythmia. In such clinical applications, the drugs exert their primary effect by blocking voltage-gated Na^+ channels of which mediate the upstroke of the action potential in electrically excitable cells such as neurons and cardiac myocytes (61). Thus, LA drugs suppress hyperexcitability.

However, LAs drugs are also known to block certain K^+ channels. For example the LA drug bupivacaine blocks the mammalian Kv1.5 channel and the cardiac hERG K^+ channel with IC_{50} values (the concentration that inhibits 50% of the K^+ current) of 9 μM and 20 μM, respectively (62). The cardiac hERG channel is also known to be particularly sensitive to promiscuous block by diverse clinical drugs apparently due to high affinity for drug binding of the central cavity of this particular K^+ channel (63-69). Such drug interactions with the hERG channel can be dangerous because suppression of hERG current may result in an acquired LQT syndrome similar to genetic defects of LQT2 described above. Block of an atrial K^+ channel, Kv1.5, by another organic cation drug such as quinidine has been found to result in rapid internalization of the channel (70)— as if binding of the drug molecule destabilized the structure of the channel and promoted degradation and turnover of the channel protein. The pore domains of mammalian K^+ channels such as hERG and Kv1.5 are structurally homologous to the KscA tetramer. Thus, study of the drug-dependence of tetramer stability of KcsA may provide insight into important pharmacological interactions in addition to their intrinsic interest for basic research.

Starting from the hypothesis that organic cations may affect tetramer stability of K^+ channels in a K^+ dependent fashion, we pursued three aims in this project: 1. Analyze structural features of the protein contact interfaces of the KcsA tetramer, 2. Investigate the effect of lidocaine and tetracaine on tetramer stability of KcsA, and 3. Investigate molecular interactions of lidocaine and tetracaine with closed and open states of the KcsA tetramer by computer simulation of drug docking to the central cavity using techniques of Monte Carlo energy minimization.

2. METHODS

Biochemical methods for expression and purification of KcsA and assay of tetramer stability by SDS-PAGE have been previously described in detail (42, 46, 47, 77). Computational procedures used for simulation of lidocaine and tetracaine docking to KcsA crystal structures and associated energy calculations are outlined in the following sources (71-77).

3. RESULTS

3.1 Structural Analysis of the Monomer-Monomer Interface of the KcsA Tetramer

Our previous results have implicated selective binding of K^+ to the selectivity filter region comprised of residues $T_{75}VGYGD_{80}$ as an important component of structural stabilization energy of the KcsA tetramer (46, 47). This finding is consistent with atomic interactions observed in KcsA crystal structures which show that each K^+ ion in the 4 S1-S4 sites of the filter is coordinated by 8 protein oxygen (O) atoms, with 4 O atoms lying in a plane above and 4 O atoms in a plane below the K^+ ion (35-37). These K^+—O interactions effectively bridge all 4 subunits of the tetramer by linking 2 O atoms from each subunit to an inorganic cation at the center of the tetramer interface. In the absence of K^+, mutually repulsive interactions of these 20 electronegative oxygen atoms would be expected to destabilize the structure of the filter. This prediction is borne out by crystallographic results showing that the filter region of KcsA deforms into a non-conductive conformation at low K^+ concentration (37, 78).

However, other evidence suggests that K^+ binding is not the only factor that stabilizes the oligomeric structure of the K^+ channel pore domain. For example, studies of the temperature dependence showed that the midpoint temperature for 50% dissociation of KcsA tetramer is ~60 °C in the absence of K^+ with 100 mM cholineCl as the electrolyte replacement (46). Similar results were observed with 100 mM TrisCl (Tris = tris-hydroxymethylaminomethane) (46). Organic cations such as choline$^+$ and Tris$^+$ do not strongly interact with K^+ channels as demonstrated by weak blocking activity in electrophysiological experiments. Thus, cholineCl and TrisCl are not expected to stabilize the selectivity filter region of KcsA in the manner of K^+. Since many oligomeric proteins readily dissociate at 25° C in the presence of detergent when analyzed by SDS-PAGE, thermal stability of the tetramer up to 60° C in the absence of K^+ suggests that other protein-protein interactions are also likely to be involved in tetramer stability of KcsA.

To obtain further insight into the nature of the protein-protein interactions that stabilize the tetrameric structure of KcsA, published crystal structures of the protein were examined using the PISA analysis program of the European Bioinformatics Institute

(http://www.ebi.ac.uk/pdbe/prot_int/pistart.html). PISA (Proteins, Interfaces, Structures and Assemblies) uses atomic coordinates of published protein crystal structures deposited in the Protein Data Bank to calculate various structural and chemical properties of macromolecular surfaces and interfaces of quaternary (oligomeric) structures (79, 80). It includes options for visualizing the location of residues that form protein-protein interfaces. It also uses tested algorithms to classify amino acid residues of protein subunits according to whether they are accessible or inaccessible to solvent, whether they form protein contact interfaces, and whether they participate in inter-subunit hydrogen bonds or ionic interactions (i.e., salt bridges). The output of the PISA program can thus be used to investigate molecular interactions involved in stabilizing the oligomeric structure of protein complexes for crystal structures available in the Protein Data Base.

As an illustration of the PISA analysis, the left panel of Figure 5 shows a space-filling representation of PDB:1K4C which is a crystal structure of KcsA tetramer determined at 2.00 Å resolution in the presence of high K^+ concentration (37). The structure is viewed as if looking at a cross section through the membrane. The top and bottom sides of the structure respectively correspond to the extracellular and intracellular faces of the channel. The four KcsA monomer subunits are colored yellow, pink, blue, and green. This structure is a closed state conformation of the channel as determined by the narrow constriction of the pore at the inner bundle crossing of the four inner helices. The right panel of Figure 5 shows two monomers of the tetramer (colored blue and cyan). The monomer-monomer interface residues identified by the PISA analysis are colored red/green in Figure 5 (right panetl). The complementary fit of residues at the inter-subunit interface suggests that steric interactions and considerations of residue shape and size may be important in protein-protein recognition and the interaction energy that contributes to tetramer formation.

Figure 5.

Space-Filling Representation of the Crystal Structure of the Closed Conformation of KcsA Tetramer (left) and Interface Contact Area (red/green) Between Two Adjacent Monomers (right)
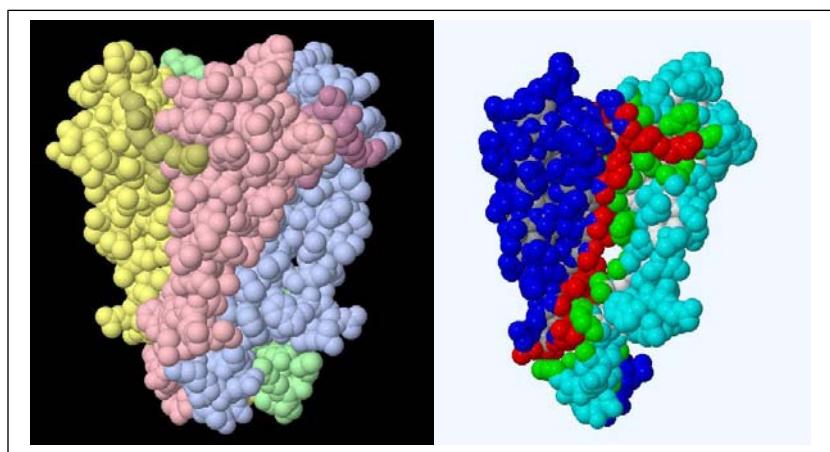
Gating of K^+ channels involves separate activation and inactivation processes that open and close the K^+-conductive pore to control the passage of K^+. As mentioned above, conformational transitions between closed and open states of the fast 'activation' process involve changes in pore diameter at the inner bundle crossing of the 4 inner helices. The actually opening and closing of the pore aperture at the helix bundle crossing is coupled to a bending motion near the middle of the inner helix that changes the shape of this helix from a linear to a kinked cylinder. For many K^+ channels such activation gating is voltage-dependent; however for KcsA, structural changes associated with such activation gating are regulated by intracellular pH (81-83). Protonation of a cluster of charged residues (not shown) located just below the bundle crossing of the inner helix increases electrostatic repulsion between these residues which favors the transition of the inner helix from linear to bent (83).

The structural changes that accompany the gating transition from closed to open are illustrated in Figure 6 which is a comparison of KcsA tetramer crystallized in closed (PDB: 1K4C) versus open (PDB: 3FB7) conformations. Figure 6 shows that the tetramer changes from an approximately conical shape in the closed state to a wider cylindrical shape in the open state due to widening of the inner pore aperture and volume expansion at the intracellular side. These conformational changes suggest that the particular residues that participate in contact interactions of the inter-subunit interface may vary in the closed vs. open state. Such changes in inter-subunit contacts could potentially lead to differences in tetramer stability for different gating states of the channel.

Figure 6.

Comparison of Closed (left) and Open (right) Conformations of the KcsA K+ Channel
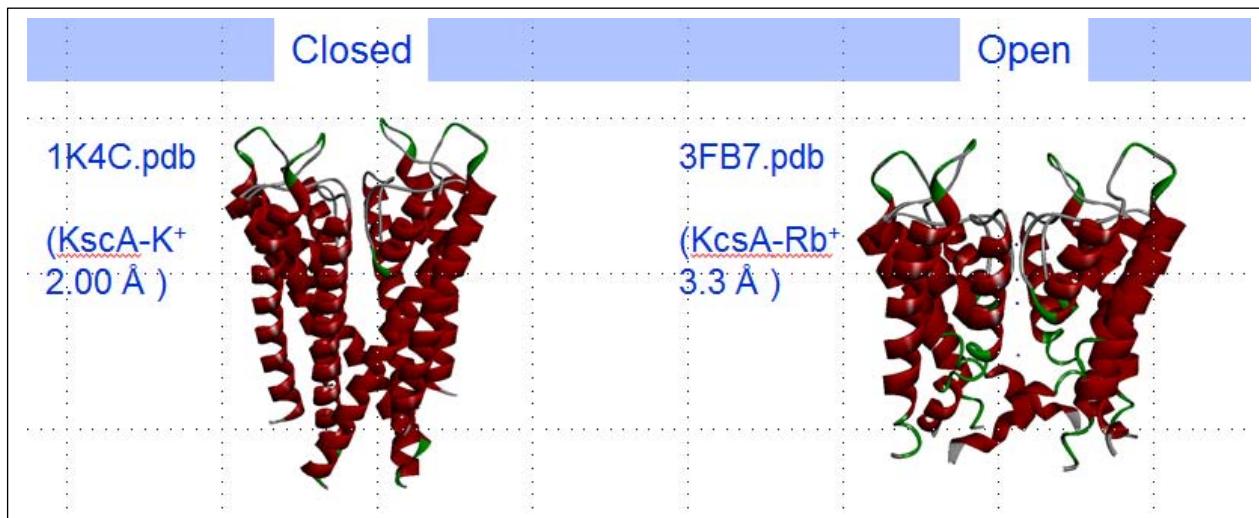
The PISA analysis program was used to address this question by comparing residues at the inter-subunit interface in the closed versus open conformation of the KcsA tetramer. The partial KcsA sequence shown in Figure 7 illustrates the location of residues determined to comprise surface area at the monomer-monomer contact interface for the structures shown in Figure 6. Each pair of sequences in Figure 7 corresponds to the KcsA sequence from the pore helix to the inner helix. This region was selected for comparison since it contains most of the residues that form the tetramer interface. The residues found to reside at the contact surface area between an adjacent pair of monomers are highlighted in yellow. The comparison of closed vs. open conformations of the channel shows that 44% more residues participate in forming the inter-subunit contact interface in the closed vs. open conformations. Specifically, the inter-subunit the contact region includes 23/26 residues at the monomer/monomer interface for the closed conformation compared to 17/17 residue for the open conformation. As expected from the bending motion of the inner helix, the majority of residues lost from the contact interface are located at the cytoplasmic end of the inner helix. As also shown in Figure 7, the PISA program predicted the location of 4 inter-subunit hydrogen bonds for both the closed and open states; however, one hydrogen bond in the inner helix is lost from the closed state (T112-V115) and replaced by a new one in the open state (T101-V106).

Figure 7.

Comparison of Contact Residues that Form the Inter - Subunit Interface for Closed vs. Open Conformations of KcsA (Residues 61-115)


```
              Closed Conformation PDB:1K4C

KcsA (61-115)   TYPRALWWSVETA TTVGYGD LYPVTL WGRLVAVVVMVAGITSFGLVTAALATWFV

KcsA (61-115)   TYPRALWWSVETA TTVGYGD LYPVTL WGRLVAVVVMVAGITSFGLVTAALATWFV


              Open Conformation PDB:3FB7

KcsA (61-115)   TYPRALWWSVETA TTVGYGD LYPVTL WGRLVAVVVMVAGITSFGLVTAALATWFV

KcsA (61-115)   TYPRALWWSVETA TTVGYGD LYPVTL WGRLVAVVVMVAGITSFGLVTAALATWFV

              --Pore Helix- -Filter -Loop- --------M2/S6 Helix---------
```

The PISA program was used to identify residues that form the inter-subunit contact interface for Closed (PDB: 1K4C) and Open (3FB7) conformations of KcsA Crystal Structures. Residues highlighted in yellow denote interface residues located within the region from the Pore Helix to the inner helix (labeled M2/S6). Solid black lines connect residue pairs predicted to form hydrogen bonds that span two adjacent monomers.

In summary, this analysis shows that the inter-subunit interface is likely to stabilize the tetrameric structure of the K^+ channel pore domain via a large surface contact area of van der Waals interactions and several inter-monomer hydrogen bonds. Since the sequence of the pore domain is highly variable among ion channels proteins that are structurally homologous to KcsA, except for the selectivity filter region, it is likely that different members of this protein family vary considerably in their intrinsic tetramer stability. Changes in the inter-subunit contact area that occur in the structural transition from the closed to the open state suggest that the tetramer stability of the open state may be significantly less than that of the closed state.

3.2 Effect of Local Anesthetics on Tetramer Stability of KcsA

As described in the Introduction, we studied the effect of two local anesthetics (LA) drugs, lidocaine and tetracaine (Figure 8), on the tetramer stability of KcsA. The blocking effect of LAs on K^+ current through the KcsA channel has not been tested, but the effect of a similar LA drug, procaine (Figure 8), on ionic currents of the squid giant axon was previously studied by the voltage-clamp technique (84). Procaine at a concentration of 4.2 mM blocked the peak Na^+ conductance (Na_V current) by 60%; whereas outward K^+ current (K_V current) was only blocked by 20% (84). These latter data correspond to IC_{50} values (concentration at 50% inhibition) of ~3 mM and ~17 mM for block of squid Na_V and K_V current, respectively. The results of these and many other pharmacological studies indicate that K^+ channels are blocked by LAs and similar hydrophobic tertiary and quaternary amine drugs with variable affinity that depends on the structure of the drug and the ion channel (62, 85, 86). Since certain organic cation drugs trigger a reduction in the functional expression of certain K^+ channels (69, 70), we hypothesized that LA drugs may affect the tetramer stability of KcsA.

Figure 8.

Chemical Structures of Lidocaine, Tetracaine, and Procaine
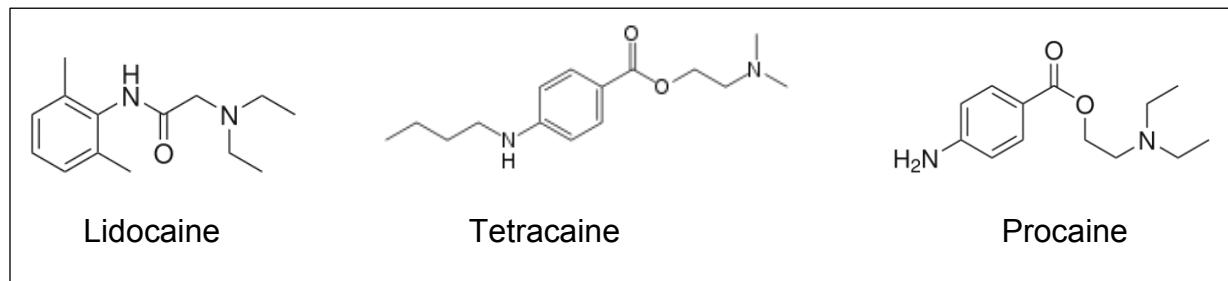
As previously described (46), KcsA tetramer is completely stable in the presence of 5 mM K^+ at least up to 99°C, but exhibits a greatly reduced $T_{0.5}$ of ~46°C in the absence of K^+ (Figure 9A, B). Results of Figure 9A show that in the presence of 5 mM K^+, lidocaine (20 mM) and tetracaine (5 mM) lower the mid-point temperature ($T_{0.5}$) for thermal stability of KcsA to 98°C and 76°C, respectively.   However in the absence of K^+, 20 mM lidocaine had no effect on thermal stability of KcsA but 5 mM tetracaine caused complete dissociation of the tetramer at 22°C or above (Figure 9B). These results demonstrate that the effect of LA drugs on KcsA tetramer stability depends greatly on the particular drug and the presence or absence of K^+.

Figure 9.

Effect of Lidocaine and Tetracaine on Thermal Stability of KcsA Tetramer in the Presence an Absence of K^+

The assay mixture for these experiments contained ~500 ng KcsA, ~6 mM dodecylmaltoside, 10 mM Hepes-Tris, pH 7.4, 100 mM cholineCl, either 5 mM KCl (A) or no added KCl (B), and either no LA (○), 20 mM lidocaine (●), or 5 mM tetracaine (△).

The effect of increasing concentration of lidocaine and tetracaine on tetramer stability of KCl exposed to 90°C for 10 minutes in the presence of 5 mM KCl is shown in Figure 10. The tetramer is completely stable under these conditions in the absence of LA drugs (Figure 9A), but increasing concentrations of lidocaine and tetracaine induced tetramer dissociation with IC_{50} values of 25 and 4.2 mM, respectively. In the absence of K^+ at room temperature (22° C), tetracaine is more effective in destabilizing KcsA tetramer with an IC_{50} value of 1.2 mM (Figure 10). These results further demonstrate that LA drugs induce ligand-dependent dissociation of KcsA tetramer as a function of drug concentration, temperature, and K^+.

Figure 10.

Effect of Increasing Concentrations Lidocaine and Tetracaine on Thermal Stability of KcsA Tetramer in the Presence and Absence of K^+

The assay mixture for these experiments contained ~500 ng KcsA, ~6 mM dodecylmaltoside, 10 mM Hepes-Tris, pH 7.4, 100 mM cholineCl, either 5 mM KCl (●, △) or no added KCl (□). Samples were incubated for 10 minutes at 90°C (●, △) or 22°C (□) in the presence of the indicted concentrations of lidocaine (●), or tetracaine (△, □).
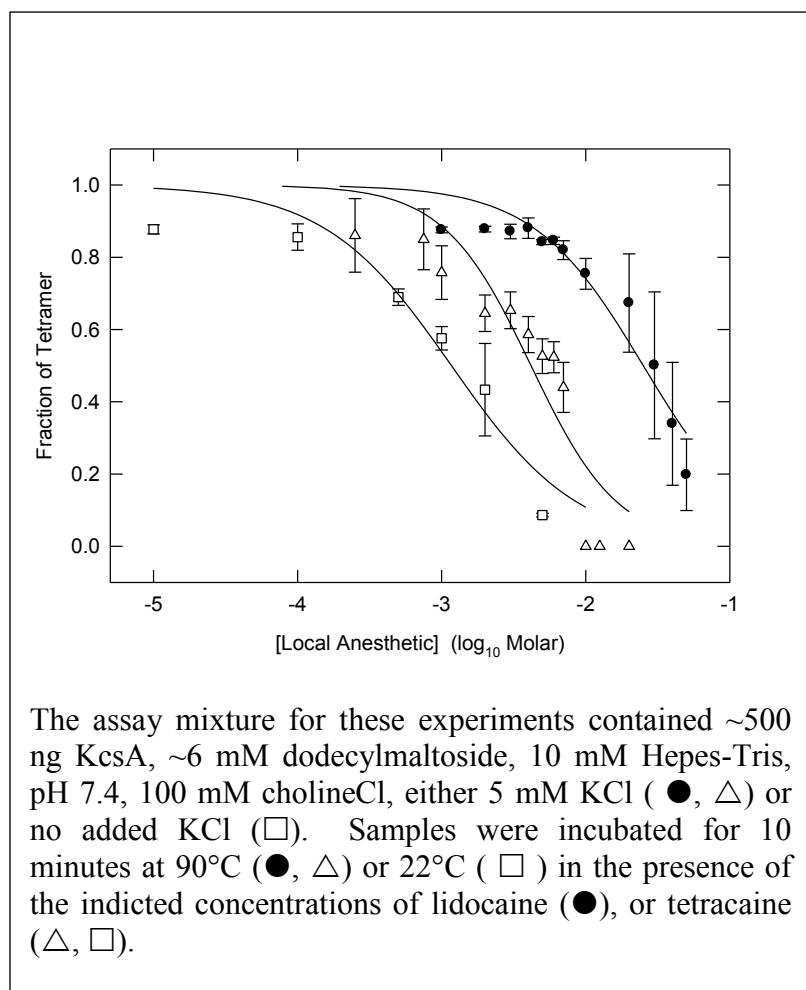
To investigate the possibility of a mutually antagonistic ligand interaction between K^+ and LA drugs, the K^+-concentration dependence of tetramer stability was studied in the absence and presence of lidocaine and tetracaine. As previously described (46, 47), increasing K^+ protects against dissociation of KcsA tetramer with a half-maximal value ($K_{0.5}$) of 1.5 mM K^+ (Figure 11). The K^+-concentration dependence is shifted to higher $K_{0.5}$ values of 6.7 and 5. 0 mM K^+ in the presence of 20 mM lidocaine and 5 mM tetracaine, respectively (Figure 11). The slope of dependence of tetramer content on K^+ concentration is also considerably shallower in the presence of LA drugs as compared to their absence (Figure 11). The experimental results of Figure 11 are consistent with the idea that LA drugs and K^+ bind to KcsA in a mutually antagonistic fashion. A possible interpretation is that binding of an LA drug in close proximity to the selectivity filter may induce the displacement of K^+ from sites the filter leading to enhanced thermal dissociation of KcsA tetramer.

Figure 11.

Stabilization of KcsA Tetramer by Increasing Concentrations of K^+ in the Absence or Presence of Lidocaine and Tetracaine

The assay mixture for these experiments contained ~500 ng KcsA, ~6 mM dodecylmaltoside, 10 mM Hepes-Tris, pH 7.4, 100 mM cholineCl, the indicated concentrations of KCl, and either no LA (○), 20 mM lidocaine (●), or 5 mM tetracaine (△). All samples were incubated for 10 minutes at 90°C before addition of sample buffer for SDS-PAGE analysis of KcsA tetramer.
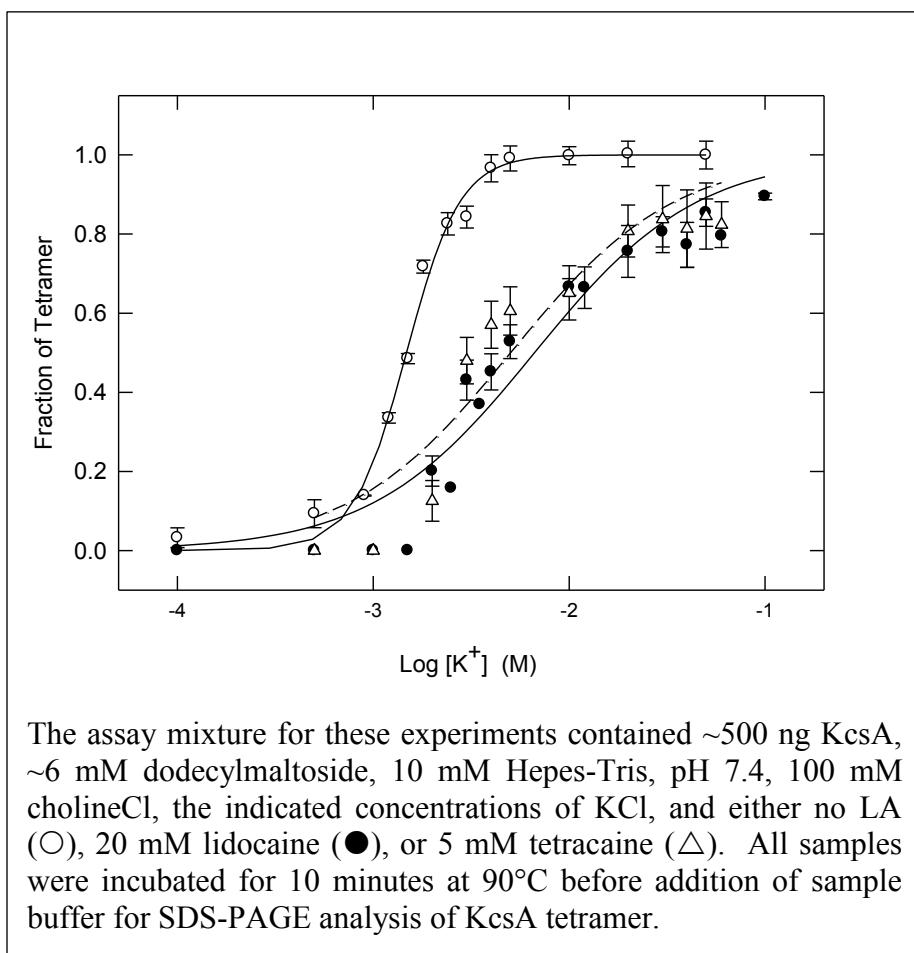
3.3 Molecular Simulations of Local Anesthetic Binding to KcsA

The preceding results suggest that binding of lidocaine and tetracaine to KcsA perturbs tetramer stability by antagonizing binding of K^+ to the selectivity filter. While many organic cations and drugs similar to LAs are known to block ionic current and alter gating kinetics of VGL channels, there is very little direct structural information on the mechanism of drug binding to these channel proteins. However, several groups of investigators have successfully solved crystal structures of KcsA in complex with bound tetrabutylammonium (TBA^+) or analogs of TBA^+ such as tetrabutylarsonium (38-41). TBA^+ is a well-known inhibitor of many K^+ channels that effectively blocks the pore from the intracellular side of the channel (85, 86). The intracellular site of action of TBA^+ suggests that this molecule enters the ion conduction pathway from the intracellular side of the channel. In confirmation of this idea, crystal structures of KcsA-TBA^+ complexes show the blocker molecule bound within the central cavity of the channel located near the middle of the bilayer just below the selectivity filter (38-41).

There are several reasons why it may be difficult to obtain crystal structures of asymmetrical molecules such as lidocaine docked to tetrameric VGL channels: 1. Binding of such molecules to an intact tetramer would break 4-fold symmetry which seems to be helpful for crystallization. 2. Possible binding of LAs to VGL channels in more than one configuration might make it difficult to obtain well-defined crystal structures. 3. Binding of LAs may destabilize the KscA tetramer under conditions appropriate for crystallization. Thus alternative approaches are needed to investigate the structural basis of drug-binding to VGL channels.

Molecular simulation and computational docking techniques have been previously used to explore possible binding modes of drug molecules to certain drug-sensitive K^+ channels such as Kv1.5 and hERG based on protein homology models (65-68, 87). However, simulations of drug binding to actual crystal structures of KcsA protein have not previously been reported. Thus, we performed molecular docking simulations in an attempt determine whether binding of lidocaine and tetracaine to the central cavity of KcsA is structurally and energetically feasible. To investigate whether drug binding might depend on the conformation of the channel, we also explored several crystal structures corresponding to closed, open, and open-inactivated states of KcsA. Energy calculations and computational procedures using a Monte-Carlo-minimization protocol are described elsewhere in detail.

Figure 12 illustrates energy-minimized docked structures of lidocaine and tetracaine bound to the central cavity using a crystal structure of the closed conformation of KcsA (PDB 1J95) (38). Similar views of the same drugs docked to an open conformation of KcsA (PDB 3PJS) (83) are shown in Figure 13. Despite the fact that these drugs are larger in size than TBA^+ (see Figure 8), both LA molecules comfortably fit within the central cavity.

Most KcsA crystal structures deposited in the Protein Data Base contain four K^+ filter ions corresponding to one K^+ ion bound in each of the S1-S4 sites of the selectivity filter. However, strong K^+-K^+ coulombic repulsion expected for simultaneous occupancy of K^+ ions in adjacent filter sites and other experimental results imply that only two configurations of two K^+ ions bound in alternate pairs of the four filter sites (with two water molecules occupying the

other sites) are possible (36, 37, 78). These two possible K^+ occupancy states of the selectivity filter are accordingly termed the S1/S3 and S2/S4 K^+ configurations. Comparison of the interaction energies of the two LA drugs docked to the open state in the S1/S3 and S2/S4 K^+ configurations reveal that binding of both lidocaine and tetracaine is more 7-8 kcal/mole more favorable in the S1/S3 vs. S2/S4 configuration (Table 1). This finding reflects greater electrostatic repulsion between the S4 vs. S3 K^+ and the protonated tertiary amine group of the drug molecule due to the closer proximity of the S4 site to the central cavity.

Figure 12.

Molecular Graphics Illustration of Simulation of Lidocaine and Tetracaine Docked to the Closed Conformation of KcsA

Lidocaine in the closed KcsA with K^+ in sites S_2 and S_4

Tetracaine in the closed KcsA with K^+ in sites S_2 and S_4 (15)
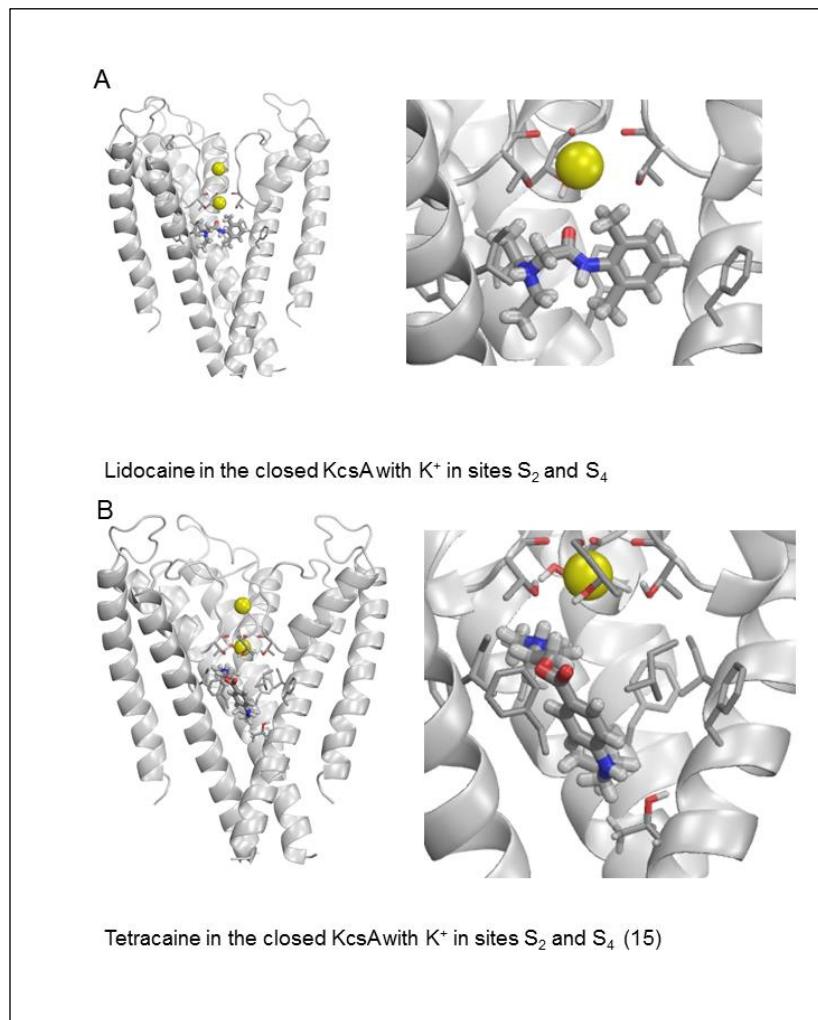
A variety of specific chemical interactions between chemical substituents of the two drug molecules and amino acid residues of KcsA were observed in the docking simulations. In the S1/S3 configuration of the open state the lidocaine carbonyl group (-C=O) exhibits favorable dipolar interactions with a water molecule at the S4 site and hydroxyl groups of Thr75 which forms the S4 site. Other residues that were found to exhibit most favorable interactions with various chemical moieties of the two LA drugs include: Thr74, Ile 100, Ser102 and Phe103 (Table 1).

Figure 13.

**Molecular Graphics Illustration of Simulation of Lidocaine and Tetracaine Docked to the Open Conformation of KcsA**

Lidocaine in the open KcsA with K$^+$ in sites S$_2$ and S$_4$

Tetracaine in the open KcsA with K$^+$ in sites S$_2$ and S$_4$
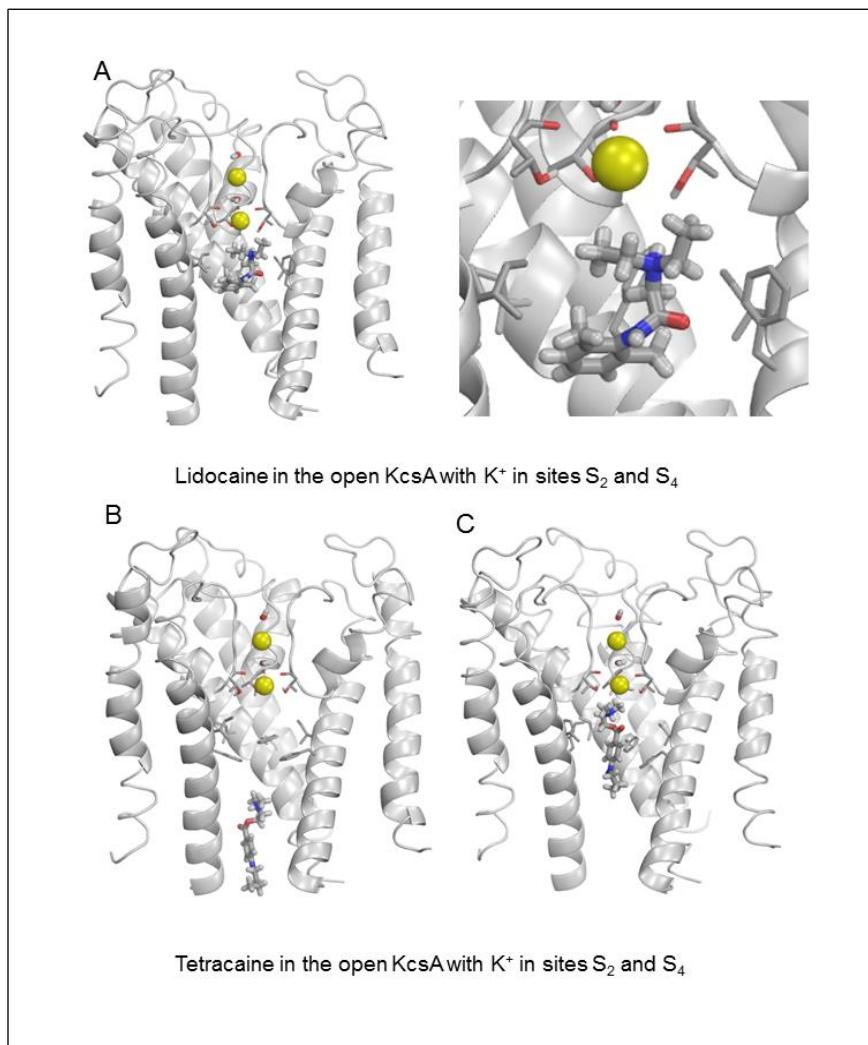
Not unexpectedly, the docking simulations suggest that LA drugs can bind to the central pore of KcsA in a variety of orientations. For example, Figure 12B shows an example of tetracaine bound near the cytoplasmic end of the pore in the S2/S4 configuration of the open channel in comparison to Figure 12C which shows the same drug molecule as an extended conformer bound close to the selectivity filer. Such results suggest that there are many possible low energy states of drug binding to the internal mouth of the ion conduction pathway. Depending on the orientation of the bound drug, there may be weak or strong electrostatic repulsion between the protonated nitrogen of tertiary amines and K^+ ions in the filter. The simulations also yielded docked states of tetracaine with the hydrophobic 'tail' of the drug partitioned within the inter-subunit interface. Such observations lend support to the idea that drug-dependent tetramer destabilization may arise from repulsive displacement of K^+ from the filter and by perturbation of inter-subunit interactions at the monomer-monomer interface.

Table 1.

Ligand-Channel Interaction Energy, Ligand-K^+ Interaction Energy, and Major Residue Contributions for Simulated Structures of KcsA Docked with Lidocaine or Tetracaine

State	Ligand	K^+ ion configuration	Drug binding energy (kcal/mol)	K^+-drug interaction energy (kcal.mol)	Major Residue Energy Contributions* (kcal/mol)			
					Phe103	Thr75	Thr74	Ile100
Open	Lidocaine	S1/S3	-23.4	0.37	-8.20	-4.15	-0.73	-2.52
		S2/S4	-15.1	2.42	-6.25	-1.61	-0.11	-2.12
	Tetracaine	S1/S3	-20.6	0.74	-2.02	-1.36	-0.72	-1.09
		S2/S4	-13.4	6.72	-9.17	-2.03	0.17	-0.75
Closed	Lidocaine	S2/S4	-19.4	0.62	-2.3	-3.64	0.04	-3.79
	Tetracaine	S2/S4	-23.1	3.51	-2.45	-3.54	-0.16	-2.86

*Other KcsA residues found to exhibit significant energy contributions in various docking modes of lidocaine and tetracaine binding are: Met 96 (-0.50 kcal/mol), Val97 (-0.61 kcal/mol), Ser102 (-0.67 kcal/mol), Val106 (-0.93 kcal/mol), and Thr107 (-3.53 kcal/mol).

4. Discussion

4.1 Findings and Conclusions

Ion channels function in the genesis and regulation of many cellular signaling processes: nerve and muscle action potentials, muscle contraction, neurotransmitter release, sensory transduction, cell growth, and immune cell activation. As discussed in the Introduction, ion channels are relevant to research problems in biodefense and bioenergy. Targeted manipulation of channel function could neutralize bacterial pore-forming toxins that are potent virulence factors in infectious diseases. Inhibition of certain ion channels may suppress runaway inflammatory responses of the innate immune system that cause life-threatening conditions such as septic shock. It may also be possible to engineer ion channels as nanobiosensors and as components of synthetic membrane materials with novel permeability and electrical properties. Such applications require detailed knowledge of structure-function relationships and deeper understanding of the molecular basis of structural stability of ion channel proteins.

Since many channels are oligomeric integral membrane proteins, the question of what holds such channels together is of fundamental interest to basic and applied science. A considerable body of literature indicates that many K^+ channels become non-functional at low K^+ concentration and/or upon exposure to certain drugs that antagonize K^+ binding to the selectivity filter. The goal of this project was to investigate this question at a molecular level using one of the most highly characterized K^+ channel proteins as an experimental model, the so-called KcsA K^+ channel of *Streptomyces lividans*.

As described in this report, the structural basis of K^+ channel tetramer stability was pursued by three approaches: 1. Bioinformatic analysis of the tetramer interface of KcsA. 2. Experimental studies of the effect of two local anesthetics drugs, lidocaine and tetracaine, on tetramer stability. 3. Computational molecular simulation of the binding of lidocaine and tetracaine to crystal structures of KcsA.

Investigation of the inter-subunit interface of crystal structures of the KcsA tetramer in closed (PBD 1K4C) (37) and open (PBD 3FB7) states was performed using the PISA software tool of the European Bioinformatics Institute. This analysis identified numerous residues that form the monomer-monomer contact region of the KcsA tetramer. Most of the interface contact residues are located in the region of the linear sequence from the pore helix to the inner helix. This finding is consistent with the fact that this region forms the inner core of the tetramer and includes the selectivity filter with 4 K^+ binding sites located at the center of the tetramer.

The analysis also showed that there is a large decrease (~30%) in the number of interface contact residues in the open state of the tetramer as compared to the closed state (Figure 7). As an approximate indication of the change in contact surface area, the PISA program found that the solvent-accessible surface area of the monomer-monomer interface is ~1,249 $Å^2$ for the closed state vs. ~588 $Å^2$ for the open state. A decrease in contact surface area for the closed→open gating transition of the channel is consistent with the change in conformation of the inner helix from a linear cylinder in the closed state to a kinked helix or bent cylinder in the

33

open state (Figure 6). This observation suggests that that the tetramer complex may become less stable when the channel opens, a prediction that might be tested by measuring tetramer stability as a function of pH, since opening probability of the channel increases as a function of decreasing pH. It would also be interesting to explore possible physiological implications of this effect. For example, decreased tetramer stability of the open state might lead to increased protein turnover and loss of channel surface density on the plasma membrane. Such an effect might be observed as a loss of functional channels during long-term electrical stimulation. A slow decrease in channel surface density might explain certain physiological phenomena such as muscle fatigue or current rundown often observed in long-term recording experiments.

Based on reported effects of drug interactions with certain human cardiac K^+ channels such as Kv1.5 and hERG, we hypothesized that binding of organic cation drugs might affect the tetramer stability of KcsA, a model K^+ channel protein. This hypothesis was tested by experiments that measured the tetramer content of a KcsA protein preparation in the presence of two different generic local anesthetics (LAs), lidocaine and tetracaine (Figure 8). Both LAs were found to enhance the dissociation of KcsA tetramer as a complex function of temperature (Figure 9), drug concentration (Figure 10), and K^+ concentration (Figure 11).

Tetracaine is markedly more effective than lidocaine in promoting thermal dissocation of the KcsA tetramer. For example in the absence of K^+, tetracaine (5 mM) caused complete loss of the tetramer at 22°C while 20 mM lidocaine had no effect on the thermal stability of KcsA under these conditions (Figure 9). In the presence of 5 mM K^+, the IC_{50} for promoting the loss of KcsA tetramer (upon exposure to 90°C for 10 minutes) was 4.2 mM for tetracaine vs. 25 mM for lidocaine (Figure 10). The higher apparent affinity for tetracaine vs. lidocaine is likely to be related to relative hydrophobicity of the two drug molecules. A similar trend was observed in animal studies of local anesthesia where tetracaine was found to be 6.7-fold more potent than lidocaine. Relative anesthetic potency of LA drugs is known correlate with bulk hydrophobicity as estimated by the octanol/water partition coefficient which is 5.1-fold greater for tetracaine/lidocaine (88).

Experiments with LA molecules also demonstrated a mutually antagonistic ligand interaction between LAs and K^+. Higher concentrations of tetracaine are required to induce thermal dissociation of KcsA tetramer in the absence of K^+ (IC_{50} = 4.2 mM tetracaine) vs. the presence of 5 mM K^+ (IC_{50} = 1.2 mM tetracaine) (Figure 2). Similarly, a higher range of K^+ concentration is required to stabilize the tetramer in the presence of lidocaine ($K_{0.5}$ = 6.7 mM K^+) and tetracaine ($K_{0.5}$ = 5.0 mM K^+) vs. the absence of LA drug ($K_{0.5}$ = 1.5 mM K^+) (Figure 3).

The experimental results with LA drug interactions clearly suggested that binding of organic cations destabilize the K^+ channel tetramer. We pursued structural insight to the mechanism of this ligand interaction by performing molecular simulation of the docking of lidocaine and tetracaine molecules to the central cavity of KcsA using well-established computational methods based on Monte Carlo energy minimization.

Results of the molecular simulations suggest a number of conclusions and hypotheses:

1. Both lidocaine and tetracaine dock within the central cavity of KcsA in a variety of conformations with similar binding energies.

2. In many LA binding configurations there are significant repulsive electrostatic interactions between the protonated cation of the tertiary amine LA drug and K^+ bound in the S4 and S3 sites of the selectivity filter. This finding is consistent with the antagonistic K^+-drug/organic cation interactions that enhance KcsA tetramer dissociation as observed in our experiments (e.g., Figure 11).

3. Specific hydrogen bonding interactions are possible between chemical moieties of bound LA drugs and various amino acid residues that line the inner pore (Table 1).

4. The conformations and energies of LA molecules docked within the inner cavity and pore vary depending on the closed or open gating states of the KcsA channel. This finding is reminiscent of the well-known state-dependence of LA interactions with voltage-gated Na^+ and K^+ channels.

5. In certain docked conformations, hydrophobic moieties of the drugs can insert into the monomer-monomer interface. Such interactions may also enhance tetramer dissociation.

Several published studies have previously identified particular amino acid residues of the Kv1.5 (70, 87, 89) and hERG K^+ channels (65-68) that affect the binding of organic cation drugs by measuring the change in drug affinity that occurs with mutation. The major results of these studies are illustrated in Figure 14 where the most important residues that affect drug binding affinity to Kv1.5 and hERG are highlighted in yellow. The sequence alignment of Figure 14 shows remarkable agreement between both Kv1.5 and hERG for this analysis. The important residues for drug affinity are located at the cytoplasmic end of the selectivity filer and the inner helix (labeled M2/S6). The conserved sequence alignment with KcsA shows that the major drug interaction residues correspond to Thr74 and Thr75 of the selectivity filter including the S4 K^+ site, and Ile100, Phe103, and Thr107 whose side chains face the internal aspect of the central cavity and inner pore. The molecular simulation results of Table 1 (also indicated in Figure 14 by yellow highlight of KcsA residues) are in good agreement with the mutational identification of the drug affinity residues of Kv1.5 and hERG K^+ channels. The close agreement between the our molecular simulation results and published experiments on structurally homologous proteins further supports the idea that organic cation /channel blocker drugs that bind in the central cavity of K^+ channels may also destabilize their tetrameric structure.

Figure 14.

Comparison of Aligned Sequences Corresponding to the Inner Core Pore Domain of KcsA and Related Human K^{+} Channels

```
                    --Pore Helix- -Filter –Loop- --------M2/S6 Helix----------

KcsA (61-115)    TYPRALWWSVETA TTVGYGD LYPVTL WGRLVAVVVMVAGITSFGLVTAALATWFV
Kv1.2(360-414)   SIPDAFWWAVVSM TTVGYGD MVPTTI GGKIVGSLCAIAGVLTIALPVPVIVSNFN
Kv1.5(466-520)   SIPDAFWWAVVTM TTVGYGD MRPITV GGKIVGSLCAIAGVLTIALPVPVIVSNFN
hERG (610-664)   KYVTALYFTFSSL TSVGFGN VSPNTN SEKIFSICVMLIGSLMYASIFGNVSAIIQ
```

Residue numbers correspond to the following aligned sequences for KcsA (Acc. P0A334), human Kv1.2 (Acc. NP_004965, human Kv1.5 (Acc. NP_002225), andd hERG/Kv11.1 (Acc Q12809). Residues identical to KcsA are colored red to indicate sequence homology. Drug binding residues of Kv1.5 and hERG identified by mutational analysis (65-68, 70, 87, 88) are highlighted yellow. KcsA residues found to exhibit the highest interaction energy with lidocaine and tetracaine in docking simulations (See Table 1) are also highlighted yellow.

4.2 Future Directions

Given its limited duration, this project achieved a number of interesting results. It produced experimental and theoretical evidence that organic cation drugs that block K^{+} channels may affect the tetrameric quaternary structure of the channel protein. This finding may have useful applications. For example, it may be used to develop a high-throughput format of a tetramer stability assay to screen for drugs that specifically inhibit or enhance the tetramer stability of certain physiologically important ion channels as drug targets. Since channel-forming proteins of K^{+} channels and other types of ions channels exhibit considerable structural and sequence diversity, such assays may identify channels that are especially sensitive to this form of pharmacological manipulation. This approach could have considerable utility in treatment of numerous diseases known as channelopathies that involve genetic defects of particular ion channels (e.g., cystic fibrosis, cardiac arrhythmias, epilepsies, etc).

In general, the idea that ligand-induced changes in structural stability of oligomeric membrane protein can be used to modulate biological function deserves further investigation. The development of more sophisticated assays to monitor this phenomenon may lead to new applications in bioscience and biotechnology. A number of techniques to monitor association and dissociation of protein oligomers could be applied to this problem. Differential scanning calorimetry (DSC) can be used to precisely monitor thermal transitions associated with subunit dissocation. Distance-dependent fluorescence assays that use appropriate spectroscopic probes may be useful in this regard. Possible techniques include fluorescence resonance energy transfer (FRET), excimer formation, and fluorescence depolarization. More sophisticated structural approaches can also be used to take this problem to a higher level. For example, alanine-

scanning mutagenesis of the K^+ channel inter-subunit interface could be used to map the detailed free-energy contributions of specific residue interactions that stabilize the tetramer. Likewise, advanced computation chemistry approaches could be used to develop theoretical approaches to the prediction of protein oligomer stability.

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