Part I
Introduction

1 Ion Channels, from Fantasy to Fact in Fifty Years[1]

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1.1 Introduction

Biologists have long recognized that the transport of ions and of neutral species across cell membranes is central to physiological function. Cells rely on their biomembranes, which separate the cytoplasm from the extracellular medium, to maintain the two electrolytes at very different composition. Specialized molecules, essentially biological nanodevices, have evolved to selectively control the movement of all the major physiological species. As should be clear, there have to be at least two distinct modes of transport. To maintain the disequilibrium, there must be molecular assemblies that drive ions and other permeable species against their electrochemical potential gradients. Such devices require energy input, typically coupling a vectorial pump with a chemical reaction, the dephosphorylation of ATP (adenosine triphosphate). These enzymes (*biochemical catalysts*) control highly concerted, and relatively slow, process, with turnovers of $\sim 100\ s^{-1}$.

Another class of enzymes, the focus of this chapter, controls the transmembrane flux of ions and other permeant species down their electrochemical potential gradients. Two types of molecules are immediate candidates for this purpose. Nature could have designed specialized carrier molecules, which first bind ions or other lipophobic species at the water–membrane interface and then diffuse across the membrane. Alternatively, transport could be carried out by channel-forming molecules, whose water-filled interiors form electrical shunts that provide essentially barrierless pathways for the transport of charged and polar species. Both types exist, and have similar design features: lipophilic exteriors, stabilizing their interaction with membranes, and polar interiors, stabilizing their interaction with charged and polar species. Selective ion channels, which can support fluxes as high as $\sim 10^8\ s^{-1}$, control biological electrical signaling. All such assemblies exhibit three crucial properties: they are highly permeable; they are highly selective; their opening and closing is exquisitely controlled. Understanding their behavior hinges on determining structure and relating it to function. A different class of selective channels exhibits physiologically equally important, but diametrically opposed behavior; aquaporins forbid

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ion passage of any kind, but allow the passage of water and, not infrequently, other small neutral species.

Hodgkin and Huxley's 1952 (Hodgkin and Huxley, 1952b) analysis of electrical activity in squid giant axon established that both Na^+ and K^+ contributed to the ionic current, and that the fluxes were opposed. It suggested channels as the ionic pathways, but it took 20 more years until Hladky and Haydon (Hladky and Haydon, 1972) definitively demonstrated the existence of ion channels, and then only in studies of the small antibiotic, gramicidin. When incorporated in lipid bilayer membranes bathed by electrolytes, it exhibited what has come to be the characteristic electrical signature of an ion channel: quantized bursts of current of variable duration arising in response to application of a transmembrane electric field.

From the time when ion channels were but a reasonable hypothesis, ever more reliable pictures have evolved. The major steps in transforming this idea from a plausible description of the biological assemblies responsible for controlling passive ion transport across membranes to established fact, thus linking structure to function, have involved great technical strides in electrophysiology, biochemistry, molecular biology, structure determination, computer power, theoretical chemistry, and bioinformatics. Three Nobel prizes, in 1963, 1991, and 2003, specifically cite studies devoted to ion channels. This overview describes important results of the past half century, beginning with one Nobel bookend, the Hodgkin–Huxley model (Hodgkin and Huxley, 1952b), that postulated independent sodium and potassium pathways, and ending with the revolution spawned by another, celebrating Agre's (Denker et al., 1988; Smith and Agre, 1991) discovery of aquaporins and MacKinnon's (Doyle et al., 1998) atomic level determination of a potassium channel's structure. Important electrophysiological, biochemical, molecular biological, structural, and theoretical tools are discussed in the context of the transition from studying systems containing many channels of varying degrees of complexity to investigating single-channel behavior. Examples are taken from a range of channel families illustrating different aspects of channel behavior: the model peptide gramicidin, the nicotinic receptor family, the voltage-regulated cation channel family, chloride channels, and aquaporins. Particular emphasis is placed on recent developments and some questions of current interest are posed.

1.2 Classical Biophysics

When electrically stimulated, a polarization wave propagates along the length of an axon. As squid giant axon is over a meter long, Hodgkin et al. (1952), using 1950s technology, could perfect the "voltage clamp" method in which an electrode is inserted in the axon interior, permitting local perturbation of the membrane potential from its resting value of -60 mV (a Nernst potential reflecting the fact that the axon interior concentrates potassium to which the membrane is selectively permeable), and the transmembrane current measured. They studied how varying both membrane potential and extracellular electrolyte composition altered electrical

response, distinguished and separately measured the currents carried by Na^+ and K^+ (Hodgkin and Huxley, 1952a) and determined their voltage-dependent kinetics. The ionic pathways were separate; each flux was activated and deactivated in response to voltage; each was electrodiffusive, controlled by its own resting potential. Properly parameterized, their kinetic model for axon behavior (Hodgkin and Huxley, 1952b) accounted for all salient features of the action potential. After a local partial depolarization, there is a large inward flow of Na^+ and the interior potential rises sharply, inverting membrane polarity; the sodium pathway then shuts down and the potassium pathway opens, restoring the resting potential, which causes it to shut down. The initial large depolarization provides the stimulus that propagates the polarization wave further along the axon. Further studies of the potassium pathway provided more detailed insight, indicating it was multiply occupied in its conducting state (Hodgkin and Keynes, 1955).

Hodgkin and Huxley demonstrated the existence of independent pathways for K^+ and Na^+ flow. They showed that the ionic fluxes were Nernst-like, due to electrodiffusive potential differences. Further studies showed membranes were selectively permeable to other ions as well, suggesting individual pathways for other physiologically important ions, i.e., Ca^{2+}, H^+, Cl^-, and HCO_3^-. By varying the composition of the external electrolyte, a pathway's relative permeability to different ions could be established, thus determining a selectivity sequence. For the axon's potassium pathway it is

$$K^+ \geq Rb^+ > Cs^+ \gg Na^+ \geq Li^+. \tag{1.1}$$

If electrodiffusion governs permeation, such relationships must reflect the underlying thermodynamics. What is the free energy change in removing an ion from electrolyte and inserting it into the transmembrane pathway? Are there general molecular level principles governing selectivity? Electrostatic design obviously permits discrimination based on ionic polarity. Eisenman (1962) noted that for the five alkali cations, only 11 of 120 possible selectivity sequences were commonly found. Why might this be the case? Could electrostatic influences dominate here as well? His answer developed from the study of glass electrodes and the thermodynamics of ion exchange. For an electrode that selects B^+ over A^+, the relative free energy for binding to glass must be more favorable than the relative free energy for hydration, i.e.,

$$G_B(\text{glass}) - G_A(\text{glass}) < G_B(\text{aqueous}) - G_A(\text{aqueous}). \tag{1.2}$$

Hydration free energy differences for isovalent ions are known. He modeled ion–electrode interaction as purely electrostatic, governed by the contact distance between the bound cation and the anionic site in the glass. For (univalent) alkali cations, the interaction energy, E, is

$$E = z_A e^2 N_{Av}/(4\pi\varepsilon\varepsilon_0[r_A + r_C]), \tag{1.3}$$

where z_A is the anionic valence, e the electronic charge, N_{Av} Avogadro's number, ε the dielectric constant, ε_0 the vacuum permittivity, r_C the cation radius, and r_A the radius of the anionic binding site. For large anions, ionic hydration (the right-hand side of (1.2)) governs the equilibrium; the electrode (or peptide) would select for Cs^+. As the anion becomes smaller, the left-hand side of (1.2) becomes ever more important, ultimately leading to selection for the smallest cation, Li^+. As the radius of the anionic site decreases, this simple electrostatic theory generates the 11 observed selectivity sequences. Nothing about the Eisenman argument is limited to glass electrodes. Similar qualitative considerations apply to binding sites along transmembrane ionic pathways and Eisenman's sequence III or IV (Eisenman, 1962) describes the selectivity of the potassium pathway (1.1). Recent work suggests that slightly modified, these electrostatic considerations could quantitatively account for selectivity in the potassium channel (Noskov et al., 2004b).

As transmembrane ionic pathways are narrow water-filled shunts surrounded by protein and lipid, electrostatics is central to understanding the influence of these surroundings. Parsegian (1969) was the first to provide quantitative estimates of how the associated permittivity differences could affect ionic transport. The energy barrier associated with ionic motion from aqueous electrolyte ($\varepsilon \sim 80$) directly into a membrane ($\varepsilon \sim 2$) is prohibitive. It is much reduced, but not eliminated, for water-filled transmembrane conduits. Even if water in the path is dielectrically equivalent to bulk water, an ion induces charges along the water–lipid interface, which impede its translocation. Were trapped water electrically inequivalent to ambient water, its ability to reorganize and shield an ion from the low ε surroundings would be reduced, thus increasing the barrier. In addition, a charging energy would be associated with ionic transfer to lower ε surroundings, also increasing the barrier. These dielectrically induced energy barriers, impeding electrodiffusion, are reduced due to interaction with Eisenman-like binding sites along the path.

Classical electrodiffusion views ion flow as movement down an electrochemical potential gradient, modulated by travel over a sequence of wells and barriers, reflecting a series of ion binding sites. This is naturally treated by biochemical kinetics, which, at its simplest, invokes a two-step translocation process: ion transfer from water to the binding well from one side of the membrane, followed by dissociation to the other side. This is mechanistically expressed as

$$\mathrm{I_L} + \mathrm{Ch} \leftrightarrow \mathrm{I \cdot Ch} \tag{1.4a}$$

$$\mathrm{I \cdot Ch} \leftrightarrow \mathrm{I_R} + \mathrm{Ch}, \tag{1.4b}$$

where $\mathrm{I_L}$ and $\mathrm{I_R}$ represent ions to the left and right of the membrane, Ch is the channel and $\mathrm{I \cdot Ch}$ the ion at the binding site. The rate of entry from the left is $R_f^L = k_\mathrm{f}[\mathrm{I_L}][\mathrm{Ch}]$ and the rate of back reaction is $R_b^L = k_\mathrm{b}\,[\mathrm{I \cdot Ch}]$; k_f and k_b are rate constants and square brackets signify species concentrations. Similar expressions describe processes occurring on the right. Analysis of electrophysiological data can establish the rate constants. Microscopic interpretation involves using these

parameters to deduce the associated energetics. The classic work of Arrhenius (1887) showed that rate constants take the form

$$k = A \exp(-E_A/RT), \tag{1.5}$$

where E_A is an "activation energy," T the absolute temperature, and R the gas constant. The topological picture identifies E_A as the energy required to surmount an activation barrier along the electrodiffusive pathway. The biochemical problem requires deconvoluting A and E_A. In ordinary chemical kinetics this is done by determining how k varies with T. It is more difficult biochemically: wide temperature ranges cannot be accessed; proteins denature; membranes undergo phase transitions. From quantum statistics Eyring (1935) developed absolute reaction rate theory where, given an energy profile, rate constants may be computed. His expression takes the form

$$k = \nu \exp(-\Delta G^{\ddagger}/RT) = \nu \exp(\Delta S^{\ddagger}/R) \exp(-\Delta H^{\ddagger}/RT), \tag{1.6}$$

where $\Delta G^{\ddagger}$ is the standard free energy change in forming the "activated complex" from reactants and $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ are the corresponding standard enthalpy and entropy changes. The activated complex is a saddle point on a multidimensional potential surface describing interacting species and ν is the frequency with which molecules in this activated state proceed to products. While the correspondence between (1.5) and (1.6) is superficially seductive, in the absence of temperature variation studies extracting activation enthalpies or free energies from (1.6) is fraught with difficulty since neither the structure reorganizational term, $\Delta S^{\ddagger}$, nor the frequency factor, ν, are easy to estimate (Jordan, 1999).

1.3 Pharmacology and Single Channels

Hodgkin and Huxley's work (Hodgkin and Huxley, 1952a,b; Hodgkin et al., 1952) was done on whole cell preparations. Distinguishing potassium and sodium currents and eliminating perturbations from other membrane components required both clever technique and a system relatively rich in sodium and potassium pathways. More general study entailed suppressing the competing currents or developing techniques for pathway isolation.

Neurotoxins, which selectively block the pathways, pharmacologically separate sodium and potassium contributions to the action potential. Tetrodotoxin (TTX), which makes fugu such a risky delicacy, eliminates the sodium current (Narahashi et al., 1964; Nakamura et al., 1965), thus isolating the potassium pathway. The quaternary amine, tetraethyl ammonium (TEA), has a complementary effect, blocking the potassium pathway (Hagiwara and Saito, 1959; Armstrong and Binstock, 1965). The discovery that each ionic pathway could be individually suppressed showed that sodium and potassium crossed the membrane by separate localized

paths (Hille, 1970). The possibility that they shared a common pathway (Mullins, 1959, 1968), with selectivity arising from small changes in pathway structure, was effectively eliminated as the dominant influence (however, there is evidence that allotropic mechanisms can occasionally affect selectivity (Callahan and Korn, 1994; Immke et al., 1999)). It further provided strong inferential evidence for the notion that these were, in fact, transmembrane channels. Most importantly, it implied these channels were molecular receptors, with toxins acting at specific sites. Viewed this way, channels are enzymes that facilitate ion flux and toxins are reversible inhibitors, described by the physical chemist's Langmuir adsorption isotherm or the biochemist's Michaelis–Menten expression. The 1972 Hladky–Haydon (Hladky and Haydon, 1972) studies on gramicidin provided final and compelling evidence for the existence of an ion channel, albeit a simple one.

The acceptance of the channel paradigm radically altered the conceptual framework, focusing interest on identifying and characterizing specific structural features, and developing putative models, "cartoons," which often bore an uncanny resemblance to the actual structures found years later. Studies by Hille (1971, 1973), with a set of organic cations, estimated the dimensions of the sodium and potassium channel pores, showing that in neither case could occlusion account for selectivity. A chemically attractive hypothesis, similar to Eisenman's selectivity theory (Eisenman, 1962), came immediately to mind, that the orifices were surrounded by rings of carbonyl oxygen atoms, regions sufficiently negatively charged to compensate for the free energy of dehydration (Bezanilla and Armstrong, 1972; Hille, 1973). Thirty years later, this idea was confirmed by MacKinnon's X-ray structure (Doyle et al., 1998) of a potassium channel. While no sodium channel structure has yet been determined, subsequent work has demonstrated that here charged residues, not carbonyl groups, control selectivity (Heinemann et al., 1992; Yang et al., 1993).

Sodium channel kinetics is complex. Whole cell studies showed that, in addition to open and closed states, there is a functionally distinct "inactivated" state. When the channel is open, Na^+ streams down the electrochemical potential gradient entering the cell, toward a locally negative region. But the channel does not long remain conducting. It shuts down, and requires potential reversal to slowly undo inactivation. These kinetics are consistent with reversible block of the pore's inner mouth by a positively charged group, which Armstrong intuited acted like a "ball and chain" (Armstrong et al., 1973), a large tethered group that swings into the pore's inner mouth when it becomes negatively charged. Work of the last 30 years has confirmed this hypothesis: large organic cations, injected into the cell, immobilize recovery from inactivation (Yeh and Armstrong, 1978); excision of the "ball and chain" domain, eliminates inactivation (Hoshi et al., 1990; Zagotta et al., 1990). Channel opening and closing modify structure somewhere along a permeation pathway; in inactivation a bulky charged group occludes a channel entrance.

Inspired pharmacology clearly provides insight at the molecular level. But, once gramicidin was proved to be an ion channel (Hladky and Haydon, 1972), the race was on to isolate individual channel proteins, reconstitute them in bilayer membranes and make single-channel measurements free from electrical interference

by other channels. Two groups reported success in 1976 (Miller and Racker, 1976; Schein et al., 1976), totally changing the experimental landscape. Even though whole cell measurements made accurately enough can in principle provide even more information than single-channel studies, the complexity of a membrane mosaic makes teasing this out unfeasible. Thus, if a single-channel conductance is sufficiently large, the pharmacological techniques developed to study whole cell preparations are immediately transferable, permitting study with greater accuracy and in more detail.

The discovery of ClC chloride channels provides striking evidence of the value of single-channel studies. Unitary cation conductance measurements showed that there is a unique ion pathway associated with each channel protein. Chloride channel current records were strikingly different. They could not be rationalized in terms of a single ionic pathway (Miller, 1982). The data implied each protein had two identical pathways opening in separate fast processes and an additional slow step activating the whole dimer. The system behaved like a double-barreled shotgun. This remarkable cartoon was confirmed 20 years later, by X-ray structure determination (Dutzler et al., 2002).

1.4 Patch Clamp, Sequencing, and Mutagenesis

Isolation of single-channel proteins by biochemical separation methods is difficult and laborious. Electrophysiology on whole cell preparations is severely limited in the systems that can be studied. In 1976, Neher and Sakmann, in work honored by the 1991 Nobel Prize, reported a way to observe single-channel currents from tiny patches of living cellular material (Neher and Sakmann, 1976), a technique that was refined to permit fusing cell membranes with the tip of a micropipette (Hamill et al., 1981). The contents of the pipette bathing the membrane surface could be adjusted at will; the patching protocol could expose either membrane surface to this electrolyte. Thus single-channel recording became simple, accurate and reliable, and there was no limit to the cells that could be studied.

Patch clamp recording methods gave unprecedented freedom in assessing how channel function was altered by different stimuli. But mechanistic understanding was still limited to cartoon models. Channel proteins are linear arrays comprising up to a few thousand amino acid residues. They fold and form channels, but what groups give rise to ionic pathways, which to the assemblies that respond to gating stimuli, and which are responsible for channel selectivity? New tools were needed to relate structure and function. These were soon forthcoming. Numa and coworkers, applying recombinant DNA techniques to the acetylcholine receptor (AChR) channel of the electric eel, developed efficient methods to determine its primary amino acid sequence (Noda et al., 1982). This assembly, formed from five similar subunits (Raftery et al., 1980) plays a crucial role in neuromuscular transmission. Located at synaptic junctions, it governs nerve–muscle communication. The subunit sequences exhibited considerable homology, i.e., substantial residue similarities

(Noda et al., 1983). Coupling these results with rules for identifying secondary structural elements (Chou and Fasman, 1978) and regions of hydrophilicity (Hopp and Woods, 1981), provided the first solid hypothesis for channel protein architecture (Noda et al., 1983).

The AChR sequences from both calf and electric eel are substantially homologous, suggesting that the similar regions are functionally important. Using cloning techniques Numa's group was able to create a hybrid AChR, mixing subunits from the two organisms (Takai et al., 1985). In a collaboration with Sakmann, its electrical properties were measured; the hybrid and its parents had the same unitary conductance strongly suggesting that channel conductance was controlled by a string of 22 amino acids, highly homologous in each of the five subunits (Sakmann et al., 1985). By selectively mutating specific residues in the protein they confirmed this speculation (Imoto et al., 1988). The era of site-directed mutagenesis had arrived.

These tools form the everyday arsenal of modern electrophysiology. The patch clamp permits near total control of the environment in studying ion channels. Site-directed mutation permits near complete freedom in protein design. Their applications showed that the cation channels—sodium, potassium, and calcium—form a superfamily. Na^+ and Ca^{2+} channel proteins are single stranded, linking four similar peptides; K^+ channel proteins are homologous, with sequences similar to individual Na^+ or Ca^{2+} peptide subunits (Jan and Jan, 1990). Clever thermodynamics connected these observations, showing K^+ channels to be tetramers (MacKinnon, 1991). But what makes them unique? Primary sequence comparisons identify "signature" domains that control selectivity; these regions are residue stretches common to, e.g., all K^+ channels, etc. In K^+ channels, the conserved feature is a five-peptide sequence (Yellen et al., 1991). In Ca^{2+} channels the signature is a set of four negatively charged residues, one from each subunit. This filter is remarkable, favoring passage of Ca^{2+} over Na^+, even though the latter is $\sim$100 times more prevalent physiologically. Na^+ channels have residues with a net –1 charge in place of the four negative signature residues of Ca^{2+} channels. Mutational signature interconversion makes a Na^+ channel selective for Ca^{2+}, and vice versa (Heinemann et al., 1992; Yang et al., 1993).

Selectivity gives channels chemical individuality. Gating, which can be coupled with permeation in anion channels (Pusch et al., 1995), provides functional control. A branch of the cation superfamily is voltage regulated, with the sensor a highly charged domain. Unlike selectivity, which is basically understood, molecular details of gating are still controversial. Like the tale of the blind men and the elephant, different experiments suggest different structural interpretations. In one picture the sensor snuggles up to countercharged regions of the protein (Bezanilla, 2002; Gandhi and Isacoff, 2002; Horn, 2002). Another suggests the sensor is on the outer side of the assembly, attracting water to stabilize its charges, and that gating involves large sensor movement (Jiang et al., 2003b). While the jury is still out, recent work favors smaller sensor motions (Chanda et al., 2005; Posson et al., 2005; Revell Phillips et al., 2005).

Patch clamp, sequencing, and mutagenesis provided much functional information. However, the structures inferred were still cartoons. The 15-residue, channel-forming peptide gramicidin is small, amenable to NMR structure determination (Arseniev et al., 1985). Long before X-ray structures of channel proteins were available, designed chemical mutation of this cation selective channel provided detailed insights connecting structural modification to changes in channel behavior (Koeppe and Anderson, 1996). Slight sequence variations can have major consequences, making the channel voltage sensitive (Durkin et al., 1993) or totally altering its fold (Salom et al., 1995).

1.5 Structure

With the exception of gramicidin, atomic resolution structures were unavailable until 1998. Channel portraits were either cartoons or silhouettes. The histories of gramicidin and AChR are illustrative. As gramicidin has only 15 residues, conformational analysis combined with secondary structure deduction tools (Chou and Fasman, 1978; Hopp and Woods, 1981) might possibly suffice for reliable structure determination. The original hypothesis, a head-to-head, left-handed β-helix, both sufficiently long to be membrane spanning and sufficiently narrow to be valence selective, was very close to the mark (Urry, 1971). A stable channel arises when carbonyl and amino groups ~~of~~ six residues apart form hydrogen bonds to one another. However, the screw is right-handed as shown by Arseniev's 1985 pioneering NMR study (Arseniev et al., 1985). Cation selectivity arises from interaction with channel lining carbonyls, Eisenman's selectivity sequence II (Eisenman, 1962). Since gramicidin is readily deformable its structure quite sensitive to its surroundings. Comparison of the channel's structure in SDS micelles with that in oriented DMPC bilayers suggests that lipids may play important roles in channel stabilization. Channel pitch and the orientation of one of the Trp groups anchoring the channel to the water–membrane interface (surprisingly, the one furthest from the interface) are both environmentally dependent (Ketchem et al., 1997; Townsley et al., 2001).

Author: Please define SDS.

Author: Please define DMPC.

AChR, responsible for communication at the synaptic junction, is a pentameric channel formed by self-assembly of four different, but homologous, peptides with stoichiometry $\alpha_2\beta\gamma\delta$. Its structure is important not just for itself but because it is a representative of a ligand-gated superfamily of neurotransmitters that includes the glycine, glutamate, and $GABA_A$ receptors. Oriented two-dimensional preparations have been studied for 20 years by electron microscopy (EM). From the outset they portrayed gross channel architecture (Brisson and Unwin, 1985; Mitra et al., 1989), but were inadequate to reliably determine the molecular architecture of the interface between the protein and the water-filled channel constriction. Even exceptionally high (4 Å) resolution data (Miyazawa et al., 2003) might still have been inadequate for unambiguous structural inference. However in 2001, Sixma's group obtained a high-resolution X-ray structure of a related polypeptide; the acetyl choline binding protein (AChBP) (Brejc et al., 2001); its sequence is highly homologous to the N-terminal

domain of the α-subunit of AChR and provides a template facilitating interpretation of cryo-EM data, leading to a definitive picture of AChR. The importance of the AChBP structure as a surrogate for higher resolution is clearly demonstrated in studies of water channels, the aquaporins. There are significant, resolution related, differences between cryo-EM results, at 3.8 Å (Murata et al., 2000), and X-ray determinations, at 2.2 Å (Sui et al., 2001). Cryo-EM's advantage is the small size of its samples; the corollary is a limit to the number of images that can be acquired. Without guidance from AChBP, it is questionable whether cryo-EM would have solved the AChR problem.

Analysis of the AChR structure poses an interesting interpretive problem. It has been imaged in both open (Unwin, 1995) and closed states (Miyazawa et al., 2003; Unwin, 2005); in each instance the pore radius is sufficiently large to permit ion passage, with constriction diameters of ∼7 and ∼3.5 Å respectively. Why won't small cations like Na^+, K^+, and Ca^{2+} flow through the narrower constriction? Certainly not, because it is occluded. The interior of the pentameric channel is formed of ∼20 rings of predominantly nonpolar residues, which present a hydrophobic surface that would repel water from the channel's interior making it inhospitable to ions. However, two of these rings are negatively charged; these could attract cations, which could permit ionic entry into the pore and possibly cause binding and block. Theoretical analysis provides a clear pathway to discriminate between these options.

In 1998, MacKinnon solved two daunting problems: isolating and purifying channel proteins "in bulk" and crystallizing them. X-ray methods, with their superior signal to noise ratios, were then applied, determining the structure of an ion-occupied bacterial potassium channel, KcsA (Doyle et al., 1998). It completely substantiated 40 years of cartooning. The selectivity filter, formed of residues identified a decade earlier (Yellen et al., 1991), is a narrow cylinder near the channel's external mouth, ∼15 Å long, just adequate to accommodate two potassium ions easily; further in is a water-filled pool, with a third ion, all substantiating the prediction of multiple occupancy (Hodgkin and Keynes, 1955). Ions in the filter are stabilized by interaction with carbonyl oxygens of the filter residues. This lining, in some ways reminiscent of gramicidin, is only possible because the two glycines of the filter have more folding options than any other residues. However, dipolar orientation of the carbonyls in gramicidin and KcsA differ substantially: in gramicidin they parallel the channel axis; in KcsA they are perpendicular to it. A pair of membrane spanning helices from each KcsA subunit surrounds the central water pool; the N to C orientation of short pore helices surrounding the filter stabilizes the central cation. Both the filter fold and the aqueous cavity were utterly unexpected.

KcsA was crystallized in a nonconducting state, its interior end too narrow to permit ion passage. Another bacterial potassium channel, MthK, was trapped with its interior mouth open (Jiang et al., 2002a). Even this coarse grained picture (only the C_α coordinates could be resolved) provided further insight into K-channels, again consistent with electrophysiological canon; it suggested strongly that a glycine residue was the gating hinge (Jiang et al., 2002b). These channels are proton regulated and calcium activated respectively. A snapshot of a third bacterial potassium channel,

KirBac1.1, shines a different spotlight on how structural details influence channel behavior (Kuo et al., 2003). The filter region replicates that of KcsA. But this channel is inwardly rectifying; in its open state, permeating cations flow preferentially into the cell. Mutational analysis of eukaryotic inward rectifiers implicated three residues as crucial for inward rectification (Lu and MacKinnon, 1994; Yang et al., 1995). As expected from electrophysiological inference, homology analysis shows two of these form rings of negative charge in an intracellular C-terminal domain where they can strongly attract polyvalent cations to impede outward potassium flow.

The three bacterial potassium channels share a common feature; they have two transmembrane helices, not the six characteristic of voltage-gated assemblies. In 2003, MacKinnon crystallized a thermophilic voltage-gated bacterial K-channel, KvAP (Jiang et al., 2003a). Its two interior helices, which surround the filter, differ little from those just discussed. As expected, the paddle-like voltage sensor was sited external to and quite independent of the filter assembly. However, its orientation with respect to the interior domain was significantly at odds with years of experimental inference. Instead of the sensor's four basic residues facing the filter, they abutted the lipid, a structure that generated immense interest and a firestorm of controversy (Gandhi et al., 2003; Laine et al., 2003). Why the differences? A voltage sensor, by its very nature is balanced on a hair trigger; crystallization may have severely reoriented it. Alternatively, eukaryotic and prokaryotic organelles (cells with and without nuclei) might differ fundamentally. EPR studies provided structural evidence to bridge the gap (Cuello et al., 2004); the sensor's charged groups interface with the lipid, but are oriented in a fashion that shields the charge. Recent structural work from MacKinnon's lab characterized the first eukaryotic voltage-gated assembly (Long et al., 2005a,b). This channel from the Shaker family, which forms the basis of most electrophysiological voltage-gating studies, was crystallized with lipid present, quite different (and much milder) conditions than those needed to stabilize KvAP. Its sensor paddle also floats freely but the charge group orientations no longer affront years of electrophysiological study. Two of the four basic groups nestle up to the filter assembly and the other two abut the lipid; while there are still some differences, this picture goes a long way toward reconciling structural, spectroscopic, and biochemical studies.

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A bacterial ClC chloride assembly suggests there can be important differences between prokaryotes and eukaryotes. The crystal structure of one such ClC (Dutzler et al., 2002) confirmed Miller's cartoon with its two identical parallel pathways (Miller, 1982). Another structure indicates how chloride ions get into the path, but not how they exit (Dutzler et al., 2003) and suggests that the fast gate reflects a small conformational change involving a single residue, a strictly conserved glutamate of the selectivity filter. The bacterial protein exhibits signature regions characteristic of chloride selectivity, but its conductance is too small for single-channel analysis. In fact, even though its protein is significantly homologous with that of eukaryotes, it is not a channel but a pump with exceptionally high turnover. For every two chloride ions that pass in one direction, a proton goes the other way (Accardi and Miller, 2004). The turnover is a thousand times faster than a typical pump and 100 times

slower than a channel. Might it be a missing link, which could provide insight into subtle differences that interconvert pumps and channels? Recent results show that some eukaryotic members of the ClC family are also antiporters (Picollo and Pusch, 2005; Scheel et al., 2005).

Ion channels are selective shunts promoting electrical activity. Aquaporins are just as selective, but toward a different end. Instead of catalyzing ion passage they rigorously forbid it, while allowing high fluxes of water and other small neutral, polar species. The puzzle is how a narrow water channel can totally discriminate against proton flow. Experience with gramicidin suggests that water readily forms hydrogen-bonded chains in narrow pores, alignments conducive to proton transfer along a proton wire via a Grotthus mechanism (Pomès and Roux, 1996, 2002). What makes aquaporins different? Their structures are built on a few recurring motifs (Fu et al., 2000; Sui et al., 2001; Harries et al., 2004). There are two strictly conserved sequences (asparagine–proline–alanine, NPA) near channel midpoints. About 10 Å distant is a constriction (the selectivity filter, SF), with a conserved arginine. Regions ∼10 Å to either side of the NPA domain form the narrow pore, beyond which are wide vestibules. One face of the pore lining is hydrophobic and the other is formed of oriented carbonyls, the oxygens of which point in opposite directions to either side of the NPA domains. This structural feature might specifically impact proton flow since the dipolar inversion would promote water inversion to either side of the NPA, which could break a water wire. A number of these channels' electrical features might account for their general ability to reject ions: the narrow region contains positively charged residues, oriented helix dipoles, and the conserved arginine and asparagines, all of which could deter cation passage; negatively charged inner vestibule groups and the constriction's carbonyl oxygens could deter anion entry into the pore. Theory provides ways to analyze these options in detail.

1.6 Spectroscopy

Crystallography provides unparalleled structural detail. However, the pictures are static and channel stabilization sometimes requires aggressive biochemical intervention, which may massively perturb a native structure (Zhou et al., 2001; Jiang et al., 2003a). Electro-optical methods, that correlate conductance and spectral properties, go a long way toward filling this gap (Mannuzzu et al., 1996; Cha and Bezanilla, 1997). While these approaches provide a less detailed picture than crystallography, the structural modifications needed to render proteins spectroscopically active are much milder. Coupling spectroscopy with electrophysiology is an ideal way to investigate dynamic behavior, and provide insight into details of voltage sensor motion.

In the voltage-gated cation channel superfamily (Jan and Jan, 1990), potassium channels are tetrameric (MacKinnon, 1991) while the single-stranded sodium and calcium channel proteins are built from four internally homologous repeats, each strongly resembling a potassium channel monomer. Their hydrophobicity patterns

indicated that each repeat (and the K-channel monomer) contains six α-helical regions separated by linkers. One of these, denoted S4 and universally present, has an amino acid composition $([R/K]XX)_n$, i.e., a run of up to eight arginines (or lysines) each separated by two apolar residues. Electrophysiological work indicates that the gating process controlling channel opening requires moving ∼12–16 charges through the transmembrane electric field (Schoppa et al., 1992; Aggarwal and MacKinnon, 1996); the discovery of this highly charged domain immediately suggested identifying it as the channels' voltage sensor. Ingenious mutational experiments demonstrated that the arginines did indeed move as the transmembrane voltage changed, as expected for a voltage sensor. The mutation R1448C in the channel $Na_v1.4$ was studied. This arginine, located in repeat IV, was expected to be on the channel's extracellular side. The cysteine, with its exposed SH group, readily reacts with methane thiosulfonate (MTS) moieties (Akabas et al., 1992; Karlin and Akabas, 1998). When designed with permanent charges, which cannot cross the membrane, they can be used to rigorously establish whether processes occur *cis* or *trans*. The R1448C mutant reacts with extracellular MTS reagents, but not intracellular ones. Even more importantly reaction is voltage sensitive. The Cys is only accessible if the channel is depolarized but nonreactive if the channel is hyperpolarized, just as expected, since the positively charged S4 segment would move outward in depolarization (Yang and Horn, 1995). Similar studies showed complementary movement of other S4 basic groups, those near the intracellular side (Bezanilla, 2000). What they fail to do is establish how much motion takes place and whether it occurs in stages or a single step.

Spectroscopy, coupled with site-directed cysteine mutagenesis, is a powerful tool for quantitatively establishing channel geometry and monitoring the detailed consequences of electrical perturbation. The cysteine's SH group is a "hook" on which to hang an almost endless array of substituents. Fluorescent dyes can be attached at targeted locations within the protein and directly monitored, whether buried in the transmembrane domain or located at a water–peptide interface, while simultaneously recording currents (Mannuzzu et al., 1996; Cha and Bezanilla, 1997). Not unexpectedly, electro-optical studies of S4 are totally consistent with MTS accessibility measurements. Correlating optical and electrical measurement yields detailed kinetic information suggesting that voltage sensor motion takes place in stages (Baker et al., 1998). Electro-optical study links voltage and spectral changes, providing dynamic insights not possible via crystallography.

The kinetics of resonant energy transfer is highly sensitive to donor (D) and acceptor (A) distances, falling off as R^{-6}. By engineering complementary fluorescent or luminescent moieties into the same peptide it is possible to measure D–A distances (Cha et al., 1999; Glauner et al., 1999). The voltage sensor in potassium channels provides a striking case. A mutation in the potassium channel sequence is expressed at equivalent sites in each of the four monomeric strands. By properly adjusting donor and acceptor concentrations, it is possible to synthesize a mutated channel with predominantly D_3A stoichiometry. If the four spectroscopically active moieties are roughly at the corners of a square, there are two possible D–A distances, differing

roughly by a factor of $\sqrt{2}$, a prediction borne out experimentally (Cha et al., 1999). Electro-optical studies mutating a series of residues in the S3–S4 linker determines the voltage dependence of a set of inter-residue distances and provides a dynamic monitor for correlating sensor motion with changes in voltage. While the behavior of the voltage sensor is still a matter of controversy (Jiang et al., 2003b; Long et al., 2005a), the most recent evidence indicates that gating occurs without large sensor displacements (Chanda et al., 2005; Posson et al., 2005; Revell Phillips et al., 2005).

A complementary approach to dynamically establishing the influence of voltage on structure relies on EPR spectroscopy. Here the label is the paramagnetic nitroxide moiety, linked to a peptide at a Cys-mutated site (Hubbell et al., 1996). EPR analysis of spin-labeled mutants provide a somewhat different perspective, establishing structural properties and mobilities of the labeled sites (Perozo et al., 1998, 2002). When applied to KvAP (Cuello et al., 2004), it clearly showed that the structure of this channel's voltage sensor is partially consistent with "traditional" and "paddle" models, and that both pictures required modification, an observation confirmed by crystallographic analysis of a eukaryotic potassium channel (Long et al., 2005a).

1.7 Theory

As experimental tools developed, so did theoretical ways to relate structure and function. Early studies, based on cartoon structures, treated channel–water–membrane ensembles as problems in electrostatics. They focused on discriminating among these pictures and gave qualitative insights (Parsegian, 1969; Levitt, 1978; Jordan, 1983). The aqueous shunt was presumed electrically equivalent to bulk water. Studies of narrow, selective channels were limited to treating reaction field effects that reflected presumed system geometries and the permittivity differences between functionally distinct domains: pore, protein, electrolyte, and membrane. Läuger presented the chemical kinetic, Eyring-like view, conductance as passage over a series of structurally induced barriers (Laüger, 1973), while Levitt considered conductance as an electrodiffusional process (Levitt, 1986) in a field created by the potential energy surface. Such treatments, when judiciously employed, can provide semiquantitative physical insight (Jordan, 1987; Jordan et al., 1989; Cai and Jordan, 1990), but they remain most useful as correlational tools.

Once a reasonable structure for gramicidin was available, its behavior became the focus for applying two powerful molecular level methods, Brownian dynamics (BD) (Cooper et al., 1985) and molecular dynamics (MD) (Mackay et al., 1984), and developing a more general electrodiffusional approach, Poisson–Nernst–Planck (PNP) theory (Chen et al., 1992; Eisenberg, 1999). PNP views ions as diffuse charge clouds, an adequate model for wide channels but problematical for narrow, selective ones (Corry et al., 2000, 2003; Edwards et al., 2002). When properly modified to incorporate ion discreteness, it can be applied to narrow channels (Mamonov et al., 2003), but its great strength, physical simplicity, is lost. For many applications it is a powerful, flexible tool, generating current–voltage (I–V) profiles for direct

comparison with data. By contrast, BD treats ions as discrete entities; it tracks their motion through a pore, also generating $I–V$ profiles. The aqueous pore is viewed as a viscous, dielectric continuum and stochastic ionic motion occurs in the potential field of the protein, frictionally retarded by pore water. Both PNP and BD impose severe dielectric assumptions, similar to the earlier electrodiffusional approaches (Levitt, 1986), ones which must be used cautiously (Schutz and Warshel, 2001). Their special strength is their ability to efficiently correlate the electrophysiological effects of alterations in protein structure and charge distribution with experimental $I–V$ data, thus providing insight into likely structural possibilities. MD is less constrained. It describes atomic level motion, governed by empirical force fields, but even now limits to computational power preclude direct determination of $I–V$ profiles. Wilson's pioneering study (Mackay et al., 1984) focused on ion–water–peptide correlations in gramicidin, concluding that water, in these confined surroundings, formed an oriented, hydrogen-bonded chain even in the ion-free channel.

With a single exception (Long et al., 2005a) crystallography has provided structures of bacterial ion channels, not the systems generally studied electrophysiologically. While the prokaryotic and eukaryotic assemblies have important sequence features in common (signatures for secondary structure, selectivity filters, etc.), their overall homology may be as low as 15%. Irrespective of the approach employed, two general strategies inform structure-based theoretical study of channel conduction. Both are speculative. The behavior of the bacterial assembly is analyzed, even though prokaryotic systems may not even be channels (Accardi and Miller, 2004), and correlated with observed behavior in eukaryotes, essentially arguing by analogy. Alternatively, bioinformatic alignment techniques (Thompson et al., 1994) provide ways to go from known bacterial structures to plausible model structures for the systems investigated in the wet lab (Corry et al., 2004). As these hypothetical eukaryotic structures, no matter how reasonable, aren't verifiable, there remains an irreducible fortuitous component to agreement (or disagreement) between computed permeation behavior and that observed experimentally.

While highly idealized, BD can provide significant insight. Studies on potassium-like channels provide a detailed view of the permeation process (Chung et al., 2002a). Open and closed state structures demonstrate that K-channels' inner mouths are very flexible (Doyle et al., 1998; Jiang et al., 2002a). BD shows that small changes in the size of the inner mouth easily accounts for the observed 100-fold spread in K-channel conductances (Chung et al., 2002b). Even though the bacterial ClC chloride assembly isn't a channel (Accardi and Miller, 2004), its pore may well still be a template for true channels and a entry for generating likely model structures for members of the eukaryotic ClC channel family. With these as input, BD studies account for observed conductance behavior (Corry et al., 2004), providing evidence for the essential validity of the models.

Although attempts have been made using a microscopic–mesoscopic approach (Burykin et al., 2002), MD hasn't yet directly reproduced $I–V$ profiles; however, there is an indirect pathway—computing a potential of mean force (PMF), in essence the permeation free energy for ion transfer from bulk electrolyte to the channel

interior. This has provided surprising insights, predicting a K-channel ion binding site (Bernèche and Roux, 2001) before experimental confirmation (Zhou et al., 2001) and generating a PMF that, when coupled with a BD treatment of field-driven diffusion, reproduces conductance measurements (Bernèche and Roux, 2003).

The origin of gating in AChR poses a challenge. Its closed pore is still quite wide, ∼3.1 Å radius (Miyazawa et al., 2003). What is the exclusionary mechanism? Very likely a hydrophobic one (Beckstein et al., 2001; Beckstein and Sansom, 2004). Water naturally tends to be expelled from the greasy interior of a narrow, nonpolar pore and ion entry is facile only if the ion is fully hydrated (Beckstein and Sansom, 2004). The critical radii, 3.5 Å and 6.5 Å respectively, closely mimic the radii of closed (Unwin, 1995) and open forms of AChR (Miyazawa et al., 2003).

Author: Beckstein, 2004 has been changed into Beckstein and Sansom, 2004 as per the reference list. Is this OK?

Atomic level modeling of permeation through ClC channels, which is coupled with the opening of the fast gate, is a knotty problem. The X-ray structures indicate that movement of the selectivity filter glutamate is needed for permeation (Dutzler et al., 2002) and strongly imply that this only occurs after it is protonated (Dutzler et al., 2003). Atomic level computations, based on modeling the bacterial pore, demonstrates this to be a plausible mechanism for ion entry from the external electrolyte (Miloshevsky and Jordan, 2003; Bostick and Berkowitz, 2004; Cohen and Schulten, 2004; Faraldo-Gomez and Roux, 2004). Modeling reproduces the observed ion binding sites and suggests the possibility that occupancy of an additional external site plays an important role in proton-assisted, fast gating (Bostick and Berkowitz, 2004). While artificially opened channels allow ionic transit (Cohen and Schulten, 2004; Corry et al., 2004), none of the analyses provides a natural mechanism for ion transit from the central binding site into the cytoplasm; the block created by the filter's serine and tyrosine residues remains impassable.

Aquaporins ability to reject most ions has been studied extensively and is readily explained as reflecting channel electrostatics. However, how a water-filled tube absolutely forbids proton passage is more controversial. If proton interaction with the channel mimicked that of other cations, electrostatics would dominate (de Groot et al., 2003; Chakrabarti et al., 2004; Ilan et al., 2004; Miloshevsky and Jordan, 2004b). However, if a water wire formed the protonic charge could be delocalized, which would reduce the electrostatic penalty for permeation. Conceivably it is disruption of a pore spanning water wire that inhibits proton passage (Tajkhorshid et al., 2002; Jensen et al., 2003); this could be induced by the reversal in carbonyl orientation to either side of the NPR motif, coupled with the influence of the NPR itself. An alternate explanation implicates two contributing factors: electrostatics and the partial dehydration of a proton upon pore entry (Burykin and Warshel, 2003, 2004). The role of the conserved arginine and asparagines is less contentious. Both are crucial determinants in excluding cations from the pore (Miloshevsky and Jordan, 2004b).

The signature property of the potassium channel filter is its selectivity. The larger ion, potassium, permeates while the smaller one, sodium, does not. Why is this? If the filter were sufficiently rigid, Born model arguments suggest that its structure would favor binding potassium over sodium (Doyle et al., 1998). All analyses suggest

that some filter flexibility is still consistent with this basic physical picture (Allen et al., 2000; Bernèche and Roux, 2001; Luzhkov and Åqvist, 2001; Burykin et al., 2003; Garofoli and Jordan, 2003). However, recent MD studies suggest the filter is so flexible that it can cradle potassium and sodium equally well (Noskov et al., 2004a), in which case an alternate explanation is needed. The answer may well be found in an extension of Eisenman's model (Eisenman, 1962), where selectivity reflects the strength of the local electric field sensed by an ion at its binding site.

But there remain problems. Gramicidin, which is structurally superbly characterized (Ketchem et al., 1997) and for which the electrophysiological data set is essentially unlimited (Koeppe and Anderson, 1996), is the critical testing ground. Here the picture is mixed. PMF computations based on standard force fields yield energy barriers implying conductances $\sim 10^7$-fold too small (Allen et al., 2003). What has gone wrong? Possibly methodological artifacts, which can be corrected for (Allen et al., 2004), or possibly the force fields (Dorman and Jordan, 2004; Miloshevsky and Jordan, 2004a). The question remains open.

Given the present limitations of high-level theory, studies of simplified abstractions are still valuable. A physical explanation of selectivity in sodium and calcium channels derives from the observations that the cation channels form a superfamily (Jan and Jan, 1990) and that exchange of signature sequences interconverts sodium and calcium channel selectivity behavior (Heinemann et al., 1992; Yang et al., 1993). The Ca channel filter has a net charge of –4 (Glu–Glu–Glu–Glu) while in the Na filter the charge is only –1 (Asp–Glu–Lys–Ala). If the residues face into an aqueous domain similar in size to that of a potassium channel's inner pore, there will be huge differences in the local electric fields. Charge compensation in a Ca channel requires residence by two Ca^{2+} or four Na^{+}; selection for calcium arises because sodium occupancy is inhibited due to crowding and additional electrostatic repulsion (Nonner et al., 2000). Extensions of the approach rationalize the calcium channel's preference for Ca^{2+} over Ba^{2+} and the effect of ion size on monovalent cation residency (Boda et al., 2001); suitably modified it also accounts for selectivity properties of the sodium channel filter (Boda et al., 2002).

1.8 What's Next?

Predicting scientific advances is a fool's errand, but irresistible. A bacterial sodium channel has been crystallized and, with good fortune, its structure will soon appear and put speculation to rest. The next few years should resolve the mechanism of voltage gating. The functional basis for differences between prokaryotic and eukaryotic ClCs should be clarified, providing insight into the structural features distinguishing channels from pumps. New theoretical tools, applicable to millisecond processes, will provide insight into gating, the slow conformational changes that control channel opening; attempts have already been made (Gullingsrud and Schulten, 2003). Channels will be engineered in novel ways, creating practical nanodevices, which has already been done with gramicidin; it is only a matter of time until similar

modifications are grafted onto biological channels. Detailed structural knowledge will lead to hosts of pharmaceutical products, specifically targeted at biological ion channels. Theory will be more prominent, providing quantitative physical explanations of the mechanisms of permeation, selectivity, and gating.

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