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Conversation between Voltage Sensors and Gates of Ion Channels

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Ion channels are transmembrane proteins that catalyze the movement of ions across the hostile hydrophobic environment of the lipid bilayer. Unlike their smaller relatives, carriers such as valinomycin (*1*), ion channels do not shuttle their cargo across the lipid; instead they create a hydrophilic pathway through which ions can diffuse, almost as easily in some cases as they move through the aqueous solutions that bathe the cell membrane (*2*). This downhill ion flux, powered by the ions' transmembrane electrochemical gradient, is exploited for a variety of cellular functions, most notably the generation of electrical signals in nerve and muscle cells. For these processes to work efficiently, the cell must maintain the ionic gradients across cell membranes. This requires energy-driven pumps, like the sodium−potassium ATPase. However, these pumps cannot by themselves keep up with the massive fluxes of ions moving through channels, especially sodium and calcium channels. So these channels must remain shut most of the time to minimize dissipating the ion gradients and only open transiently when called upon to perform some task for the cell.

The opening and closing of channels is a process called gating, and it is regulated primarily in two ways, either by the binding of ligands or by changes in transmembrane potential (*2*). The latter process, voltage-dependent gating, is the subject of this review.

The design strategy for a voltage-dependent ion channel is rather simple. A minimalist channel must have at least two conformations, closed and open, and these states must differ in the net distribution of charge across the membrane electric field. For the example in Figure 1, the central permeation pathway is shown with gates either closed (left) or open (right). The channel is depicted as having more positive charge located near the cytoplasmic surface when the channel is closed. This prototypical channel will be voltage dependent; depolarization (an increase in positivity at the cytoplasmic side of the membrane) will energetically favor the open conformation (*3*).

The channel in Figure 1 is drawn as if it had two types of moving parts, cationic voltage sensors that move in response to changes of membrane potential and gates that control access to the permeation pathway (*3*−*5*). Nevertheless, there is no requirement that voltage sensors be separate entities from gates. For example, if the gate were charged, it could move through the electric field as it opens or closes a channel. Then it would also be a voltage sensor. However, the consensus opinion is that voltage sensors and gates are separate entities in the major superfamily of voltage-dependent ion channels, and there are excellent candidates for each in specific regions of the channel protein, as described below. We even have some crude ideas about the conformational changes that underlie movements of both voltage sensors and gates. However, we are almost totally clueless about how the movement of one affects the conformation of the other, that is, how these movements are coupled.

We do not yet have even a low-resolution structure of the voltage-dependent channels selective for either sodium, calcium, or potassium ions. However, we know that they have an approximately 4-fold radial symmetry with each domain, or subunit, containing six putative transmembrane segments, S1−S6 (Figure 2). Potassium channels are made

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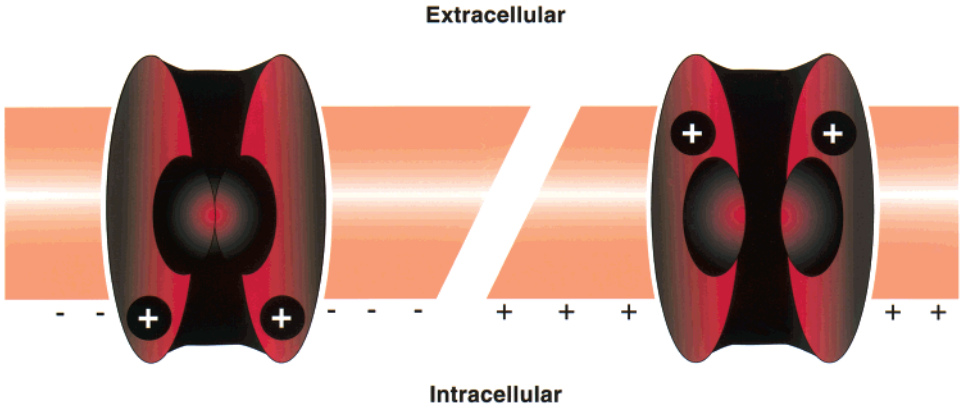

FIGURE 1: Prototypical voltage-gated channel with two conformations, closed (left) and open (right). The open configuration has more positive charge near the extracellular side of the membrane electric field than in the closed channel. Therefore, depolarization will energetically favor the open channel.

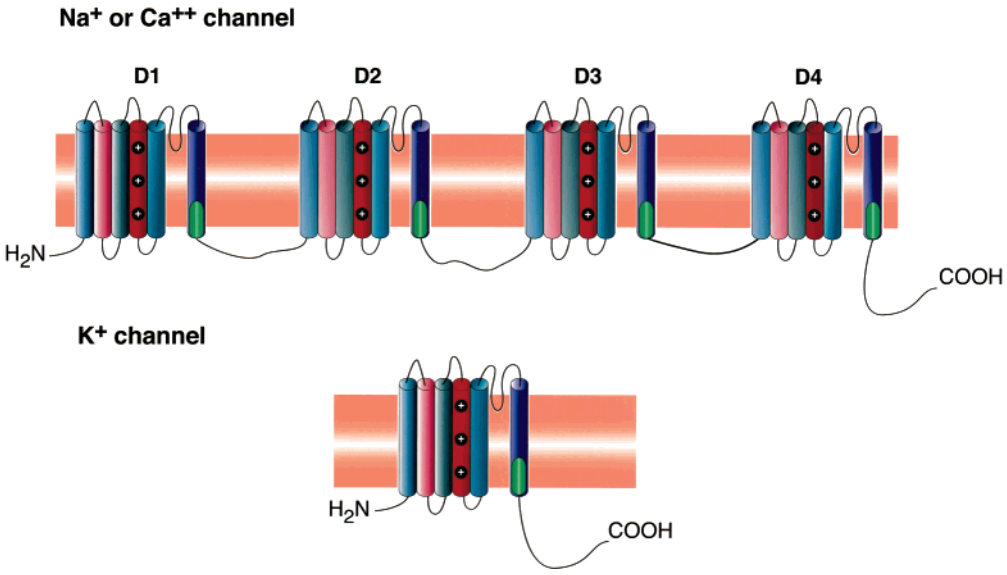

FIGURE 2: Transmembrane topology of sodium, calcium, and potassium channels. Extracellular is depicted above the membrane. Each domain or subunit contains six transmembrane segments, S1−S6. Each S4 segment (in red) has up to eight basic residues separated from each other in the primary sequence by two neutral residues. The cytoplasmic ends of the S6 segments (green) are believed to comprise the activation gate. The cytoplasmic loop between domains 3 and 4 of the sodium channel (heavy line) is the putative fast inactivation gate.

of four subunits arranged around a central permeation pathway. In sodium and calcium channels four homologous domains of a single polypeptide are arranged around the permeation pathway. The ion-selective permeation pathway is lined primarily by the four S6 segments and by the extracellular S5−S6 loops. The S5 and S6 segments along with the inclusive S5−S6 linker are sometimes called the pore domain of a subunit or domain. The main voltage sensors are the four positively charged S4 segments, shown in red. Each S4 segment has three to eight basic residues, either arginines or lysines, which are usually separated from each other by two neutral residues. Depolarization is expected to move S4 segments outward through the electric field (*3*−*8*). One early consequence of this S4 movement is the opening of the activation gate, believed to be formed by the cytoplasmic ends of the channel's four S6 segments, at the entrance of the permeation pathway (*9*−*11*). Prolonged depolarization also causes the inactivation gates, located elsewhere in the protein, to close.

Movement of Voltage Sensors. The early evidence that S4 segments were voltage sensors was based on effects of mutagenesis on voltage-dependent gating of ion channels expressed in eukaryotic cells and examined using electrophysiology. Point mutations of either charged or neutral residues of S4 segments affect a channel's ability to respond to changes of membrane potential (*12*−*14*). Such results are insufficient, however, to establish whether S4s are voltage sensors, even though they are charged transmembrane segments. They could just as well be gates or regions involved in coupling voltage sensors to gates. Another approach was needed, and two of them were developed.

The first was cysteine scanning, which we applied for examining S4 movement in 1995 (*15*) and was later exploited by several other groups (reviewed in refs *4* and *5*). The second method involved the tagging of the external ends of S4 segments with fluorophores, introduced by the Isacoff laboratory (*16*) and later employed by both Bezanilla's and Isacoff's laboratories (reviewed in ref *5*).

Cysteine scanning involves the systematic substitution of cysteine residues into regions of interest and testing whether they are accessible to hydrophilic cysteine reagents at either the extracellular or the intracellular surface of a membrane

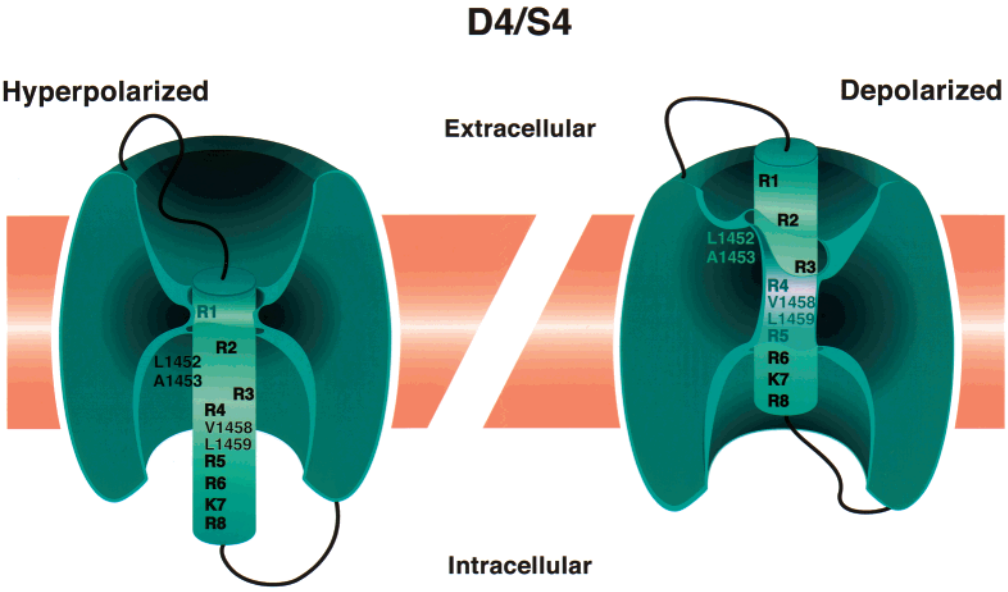

FIGURE 3: Voltage-dependent changes in accessibilities of the S4 residues of domain 4 of the sodium channel, based on cysteine scanning (*15*, *21*, *47*). The left/right panel shows accessibilities at a hyperpolarized/depolarized voltage, respectively. The cartoon represents a single domain with a central S4 segment moving through a gating pore comprised of the remaining transmembrane segments of this domain. The residues in bold (**R1**−**R6**, **K7**, **R8**) represent the seven arginine residues and the one lysine residue in this S4 segment. Each basic residue is separated from the next in the primary sequence by two neutral residues. **R1** is arginine^{1448} of the hSkM1 sodium channel. Four neutral residues are shown in the figure. Inaccessible residues are depicted with green characters; accessible residues, with black characters. Notice the large hydrophilic vestibules at either end of the S4 segment. These vestibules are required to explain the length of the inaccessible region, which must be considerably shorter than the thickness of the bilayer (40−50 Å). The accessibility pattern at the depolarized voltage, with **R1**, **R2**, and **R3** accessible but not L^{1452} or A^{1453}, is consistent with an α-helical secondary structure (*47*).

protein (*17*). Voltage sensor movement is reflected by the voltage-dependent exposure or burial of cysteines substituted into different parts of putative voltage sensors (*18*). A variant of this technique, histidine scanning, tracks accessibility by the binding and translocation of protons across the membrane (*19*). For cysteine scanning to be successful, the covalent reaction of reagents (primarily the methanethiosulfonate reagents) with cysteine must produce a significant biophysical effect on membrane currents.

One of the principal insights from cysteine scanning studies is that S4 segments, although true transmembrane structures, are almost completely accessible to hydrophilic reagents, suggesting that they are surrounded by hydrophilic crevices or vestibules, leaving only a short contact region with the hydrophobic surround (*20*, *21*). This tight ring around the S4 segment has been called the "gating pore". Changes in membrane potential appear to move the S4 segment back and forth efficiently through the gating pore (Figure 3). This movement is coupled somehow to gate movement. The short gating pore implies that only a small movement can translocate several charged residues across the membrane electric field, about three elementary charges for each S4 segment to account for the high sensitivity of these channels to changes of membrane potential (*3*, *5*). The short gating pore also intensifies the membrane electric field to levels on the order of 10^6 V/cm.

Tagging S4 segments with fluorophores has provided a different set of insights from cysteine scanning. Most of the data have been obtained on *Shaker* potassium channels expressed in *Xenopus* oocytes (*16*, *22*−*27*). Specific labeling is accomplished by cysteine substitution, using fluorophores that attach covalently to the introduced cysteines, usually by a maleimide linker. Voltage-dependent changes in the environment of the fluorophore can be detected as a change in emission intensity. Therefore, fluorescence emission can provide a dynamic readout of voltage sensor movement. The signals appear to arise primarily from quenching of emission by neighboring regions of protein, including the extracellular S3−S4 linkers. Recently, fluorescence energy transfer has been used to measure distances between S4 segments in different subunits (*28*, *29*; critiqued in ref *30*). Both of these studies estimated small (<10 Å) voltage-dependent distance changes, which were interpreted as rotational movements. For technical reasons all of these fluorescence measurements have so far been restricted to the extracellular regions of the voltage sensors.

One of the caveats to keep in mind with this method is that the fluorophores used are large (~1000 Å^3) and are attached by linkers >7 Å in length. This has two negative consequences. First, the fluorophores may themselves perturb voltage sensor movement. Second, they may not accurately track this movement. However, they do provide dynamic information about the environment in the immediate vicinity of the voltage sensors.

There are many gaps in our knowledge about voltage sensor movement. First, the efficient transfer of charge across the electric field depends critically on the topology of the vestibules that surround the S4 segment. If, as has been suggested (*28*), the S4 movement is entirely rotational, the electric field must be oriented nearly parallel to the axis of the S4 segment. The topology of the vestibules is only a speculation at present. Moreover, recent data suggest that the outward movement of positive charge is accompanied by an outward translation of the S4 segment itself (*27*); i.e., S4 movement is not completely rotational. Second, it is highly unlikely that the gating pore remains immobile during S4 movement. Histidine scanning suggests, for example, that depolarization makes the gating pore longer (*31*). Yet there

has been little effort to look for movement of the transmembrane segments that surround S4, other than the outer end of the S2 segment (*5*), the outer end of the pore domain (*5*, *27*), and S6 segments (*4*). Third, the relative orientations of the transmembrane segments that surround S4 are not known. Fourth, movement of one S4 segment might influence the movements of other S4 segments within the same channel, for either electrostatic or allosteric reasons. Yet the cooperative interaction among voltage sensors is not clear; estimates of the magnitude of S4 cooperativity differ dramatically (*23*, *32*, *33*). Finally, because the gating pore is so short and the side chains of basic residues are so long, some of the movement of charges across the membrane electric field may be due to side chain rather than peptide backbone movement. This needs to be explored to appreciate the types of S4 movements that are coupled to gate opening.

Movement of Gates. Two early experiments using internally perfused squid axons suggest that the activation and inactivation gates of ion channels are located near the cytoplasmic entrance of the permeation pathway. First, when cationic ions such as tetraethylammonium block open potassium channels from the cytoplasmic surface, the activation gates cannot close (*34*). Second, cytoplasmic proteolytic enzymes abolish inactivation of sodium channels (*35*), suggesting that the inactivation gate can be cleaved from the cytoplasmic surface of the channel.

Experiments along these lines, supplemented with mutagenesis and cysteine accessibility scanning, have confirmed the above suggestions (*4*, *36*). The most promising candidates for activation gates are the cytoplasmic ends of S6 segments, the four of which can converge to pinch off the entrance to the permeation pathway; and the so-called fast inactivation gates of sodium and potassium channels are likely to be tethered cytoplasmic pore blockers. In sodium channels the fast inactivation gate is probably the linker between the third and fourth domains (*37*). In potassium channels it is the positively charged amino terminus, one from each subunit (*38*). These four positively charged N-termini are sometimes called "inactivation particles", any one of which can block the open channel; this blocking reaction is the closing of the inactivation gate. Recovery from inactivation is equivalent to the dissociation of this inactivation particle from its blocking site in the pore mouth. Another type of inactivation, called slow inactivation for its typically slower kinetics, is likely to involve a pinching together of the extracellular end of the permeation pathway (for review, see ref *4*).

A fly in the ointment of this simplistic assignment of gates is that the selectivity filter has also been proposed to be the activation gate, or at least to be a significant part of the gate (*39*, *40*). The selectivity filter is the region near the extracellular end of the pore that accounts for the exquisite ability of these ion channels, especially potassium channels, to discriminate among small cations (*41*). If this idea has a kernel of truth, then the activation "gate" may actually be two gates at opposite ends of the permeation pathway. Presumably these gates talk to one other, perhaps through movements of S6 segments. The relative importance of these two potential activation gates is presently unknown and may differ in different types of ion channel.

This is not the only puzzle presented by gate movement. We do not know what kind of secondary structural movement (e.g., rotation, translation, tilting) causes the opening and closing of activation and inactivation gates, although there is some evidence that rotation plays a role (*42*, *43*). Furthermore, if gates move "cooperatively" (e.g., refs *44* and *45*), how do the individual parts interact? The biggest puzzle, however, is the way that voltage sensor movement controls gate movement and vice versa.

Coupling. Because of their numerous basic residues, S4 segments are likely to be insulated from lipid by other transmembrane segments. If all of the transmembrane segments are predominantly α-helical, as recent studies suggest (*46*−*49*), then all of the remaining transmembrane segments (S1−S3, S5, and S6) are needed in each subunit or domain to completely surround the S4 segment. This may be the reason voltage-dependent channels have so many transmembrane segments. This fact indicates that voltage sensor movement is equivalent to a relative movement between the S4 segment and all the other transmembrane segments (Figure 3).

With this picture in mind, two different mechanisms of coupling seem most likely. The first is that S4 movement causes a repacking of the surrounding transmembrane segments. One consequence of this cooperative movement among the transmembrane segments, especially that of the S6 segment, is the opening or closing of the activation gate. The second possibility is that when S4 segments move, they pull or twist on the two loops (S3−S4 and S4−S5) that connect them with other transmembrane segments. This tugging on the S3 and S5 segments and the resultant movement of these segments and loops would then cause the activation gate to open or close. These two mechanisms are similar in that both involve a voltage-dependent rearrangement among transmembrane segments that surround the permeation pathway. The principal difference between them is whether conformational changes in the loops play a critical role in the coupling. There is evidence for a role both of the loops and of a direct interaction among transmembrane sections in voltage-dependent gating.

Although mutations, or oxidation of introduced cysteines, in the S3−S4 linker can affect gating, the consequences are rather minor. In fact, deletion of virtually the entire linker leaves a crippled but functional channel (*50*). Therefore, the extracellular S3−S4 linker is not essential for voltage-dependent gating. This is not true for the intracellular S4−S5 linker, which is rather sensitive to mutations, some of which produce drastic functional effects (*51*−*53*). Therefore, the S4−S5 linker is a good candidate for a critical component in the process of coupling.

Recent studies also suggest an important role of interacting transmembrane segments (*49*, *54*−*56*). These studies are based on a systematic scanning of transmembrane segments, using either alanine or tryptophan substitutions, and an examination of the biophysical consequences on channel gating. The logic of this approach is that if the side chain of a residue projects into lipid or aqueous solution, channel function will tend to be less sensitive to structural alteration by mutation than if it is involved in protein packing with other transmembrane segments. The results of these studies show a pattern predicted for packed α-helices; namely, the "high impact" residues tend to be aligned along one helical face of transmembrane segments S1, S2, and S3. Furthermore, the surfaces of S5 and S6 facing away from the central permeation pathway, based on the homology between these

helices and the pore-lining helices of a bacterial potassium channel (*41*), also present stripes of high impact regions. The fact that the alanine and tryptophan substitutions can produce such profound alterations of voltage-dependent gating suggests that movements between transmembrane segments are disrupted. These postulated movements are likely, therefore, to be important links in the communication between voltage sensors and gates.

How can coupling be studied experimentally? Recently, we have been exploring the possibility of systematically immobilizing the moving parts of voltage-gated ion channels and examining the biophysical consequences on their function (*33*). To this end we designed a bifunctional cysteine reagent, benzophenone-4-carboxamidocysteine methanethiosulfonate, that will attach covalently to introduced cysteines. While recording currents from these labeled ion channels, we expose them to ultraviolet light, which causes the ketone group on the benzophenone to insert into neighboring C−H bonds, with the goal of preventing the relative movement between two secondary structural regions of the channel. We showed that this method can be used to completely immobilize either S4 segments or gates. The functional effects are already providing new insights into coupling mechanisms.

The ultimate scientific fantasy in the minds of many channelologists is to have a detailed motion picture (in Technicolor, of course!) of channels exposed to their favorite stimuli and opening or closing in response to them. A fringe benefit would be a detailed knowledge of the energetics of these coupled movements. As in a Hollywood film, one way to do this is to assemble a collection of serial, fine-grained structural snapshots. With the present technological limitations of structural biology this goal may be a long way off. However, a number of dynamic measurements of functioning channels are possible, which include electrophysiological and spectroscopic (e.g., refs *57* and *58*) techniques. As the data from a few static, structural images are combined with dynamic functional measurements, the first crude cartoons will emerge, carrying insights about molecular movement.

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