Neuron, Vol. 16, 717–722, April, 1996, Copyright ©1996 by Cell Press

A Structural Vignette Common to Voltage Sensors and Conduction Pores: Canaliculi

Review

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Ion channels catalyze the movement of ions across cell membranes by forming water-filled transmembrane pores. We know this, remarkably, in the absence of direct structural information. The observed properties of channels (high unitary transport rates, low temperature coefficients, ion–ion and ion–water flux coupling) demand an underlying pore structure (Hodgkin and Keynes, 1955; Armstrong, 1975; Miller, 1987). Now our ideas about the finer physical details of ion channels are coming into sharper focus through study of channel point mutants and their water-soluble ligands. A flurry of elegant reports suggests that channels solve the energetic problem of moving electrical charges across ~40 Å of forbidding membrane lipid by bringing the extracellular and internal aqueous compartments closer together. Gating charges and permeating ions appear to traverse channel proteins via short canals (canaliculi) of 10 Å or less. This conclusion is based on identification of channel positions (in voltage-sensing and pore-forming domains) that are separated by just a few intervening residues yet are exposed simultaneously on opposite sides of the plasma membrane of the cell.

Two reports consider how voltage-gated Na^+ (Yang et al., 1996) and K^+ channels open (Larsson et al., 1996); the remainder explore the structure of the ion conduction pathway in cyclic nucleotide–gated (CNG) cation channels (Sun et al., 1996) and voltage-gated K^+ channels (Lu and Miller, 1995; Pascual et al., 1995; Naranjo and Miller, 1996; Ranganathan et al., 1996; Gross and MacKinnon, 1996). Fully six of these studies employ one approach: substitution of a natural channel residue by cysteine to take advantage of the unique chemical reactivity of this amino acid with sulfhydryl-specific probes (Akabas et al., 1992; Stauffer and Karlin, 1994). Three studies employ peptide neurotoxins of known three-dimensional structure to map the contours of their contact sites in K^+ channel pores.

The Big Picture: Function

Ion channels are central to the electrical life of all cells (Hille, 1992). Delicately orchestrated ion fluxes through channels generate action potentials, muscular activity, and transmembrane signals. Less flamboyant, steady channel activity establishes the volume, solute concentration, and membrane potential across every viable cell. Yet, in concept, ion channel function is exceptionally simple (Zagotta et al., 1994).

$$\text{Resting} \leftrightarrow \text{Open} \leftrightarrow \text{Inactive} \qquad (1)$$

Voltage-gated ion channels exist in three functional states (equation 1). At rest they are silent. Upon membrane depolarization (perhaps from a passing action potential), the inside of the cell becomes more positive. This change in the transmembrane electric field exerts large forces on portions of channels in the membrane. Now the open conformation is energetically favored. This transition, called activation, is associated with movement of the equivalent of ten or more elementary charges completely across the membrane field for each channel (Sigworth, 1994). In the open state, ion permeation is highly selective and exceptionally efficient. K^+ channels prefer K^+ to Na^+ by 100-fold (or more) and, despite their discerning nature, can conduct 10,000,000 K^+ ions (or more) across the membrane each second through a single channel molecule. If the membrane stays depolarized, some channels remain open; however, most assume a silent inactive conformation. Channels return to rest (and are reset for action) when the membrane assumes its initial hyperpolarized potential.

The Big Picture: Structure

The cloning of genes for voltage-gated Na^+, Ca^{2+}, and K^+ channels and CNG channels revealed their membership in a molecular superfamily marked by similarities in primary sequence and predicted membrane topology (Hille, 1992). Na^+ and Ca^{2+} channels contain four homologous domains in tandem, each with six predicted membrane-spanning segments (Figure 1a). K^+ and CNG channel subunits are similar in size and topology to a single Na^+ channel domain (Figure 1b). After their cloning, protein domains that participate in specific channel functions were identified by site-directed mutation, and a crude idea of the gross molecular architecture of ion channel proteins emerged (Miller, 1991).

The S4 Domain and Channel Activation

Hodgkin and Huxely (1952) first proposed that changes in voltage might cause the movement of charged "gating particles" within nerve membranes to turn Na^+ and K^+ conductances on and off. With genes for channels now cloned and sequenced, residues in the fourth predicted transmembrane segments (S4) quickly became the leading candidates for these voltage-sensing charges (Figures 1a and 1b) (Noda et al., 1984; Catterall, 1986; Guy et al., 1986); S4 segments are marked by positively charged amino acids (arginine or lysine) at every third or fourth position, a motif unique to ion channels. Provocative reports supported these ideas (Stuhmer et al., 1989; Liman et al., 1991; Papazian et al., 1991); however, attempts to establish firmly that S4 charges mediated the effects of voltage on channel opening were inconclusive (Sigworth, 1994). This situation is now changing (Yang et al., 1996; Larsson et al., 1996).

The P Domain and Ion Permeation

These channels share an overall tetrameric anatomy. A single conduction pore is formed in each, either through pseudosymmetric folding of four homologous domains or aggregation of four independent subunits (Figure 1c). The residues linking every fifth and sixth membrane-spanning segment contribute to pore formation (P domains) and are arrayed centrally as four pore loops

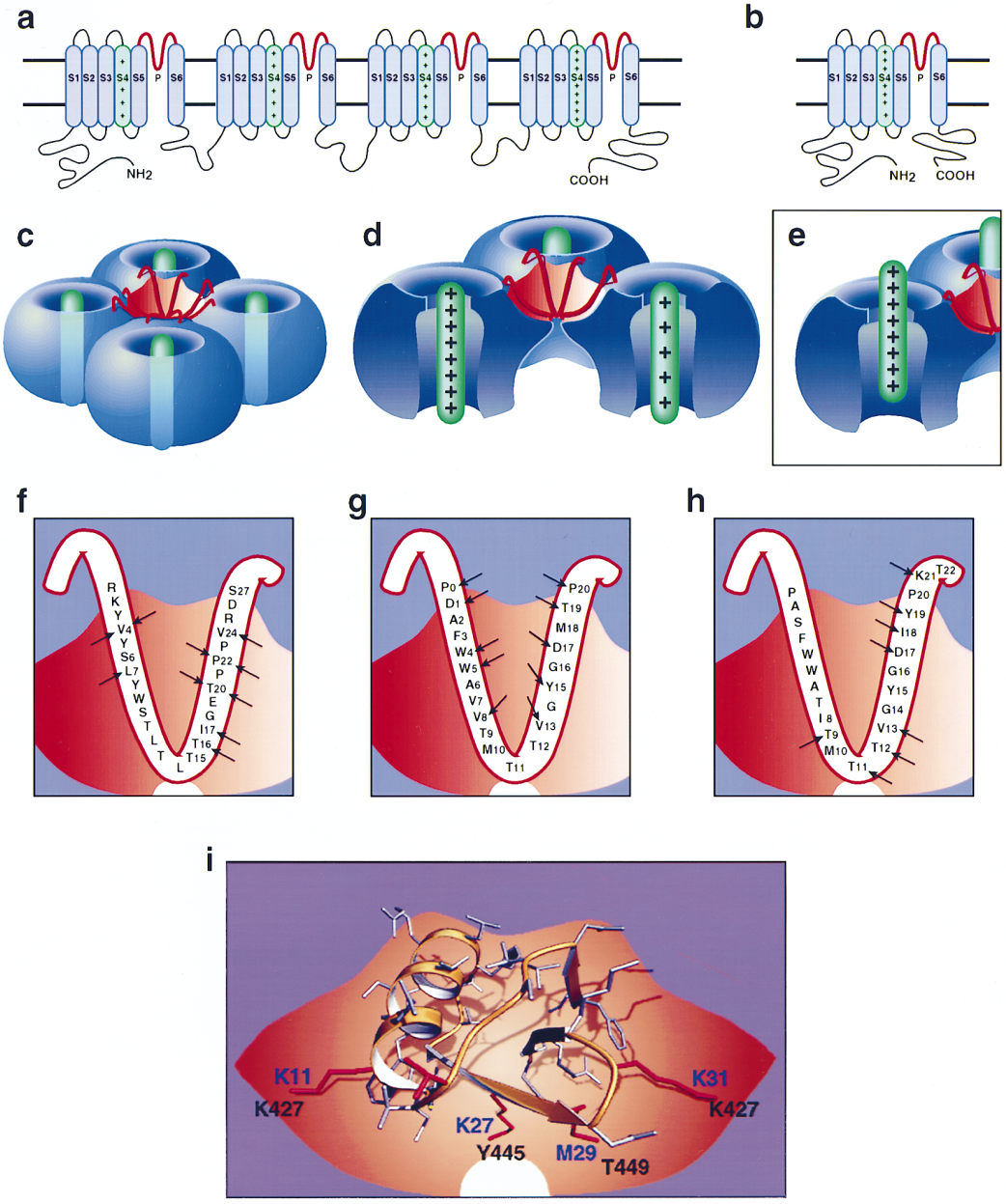

Figure 1. Proposed Ion Channel Structures

Probable membrane topology of the α-subunits of (a) voltage-gated Na^+ and Ca^{2+} channels and (b) voltage-gated K^+ and CNG channels. (c) Complete channels have tetrameric symmetry. (d) Cut-away showing a Na^+ channel with proposed S4 and conduction pore canaliculi exposed; S4 segments are depicted at rest, after Yang et al. (1996). (e) Upon depolarization, the Na^+ channel S4/D4 domain appears to move outward so that R3C is accessible to external MTSET and R4C to internal MTSET, after Yang et al. (1996). (f) CNG channel P domain residues. Numbered sites were studied by Sun et al. (1996), and those modified by external MTSEA (arrows above) and internal MTSEA (arrows below) in the closed state are indicated. (g) *Shaker* K^+ channel P domain residues. Numbered sites were studied by Lu and Miller (1995); those modified by external Ag^+ in the closed state are indicated. (h) Kv2.1 K^+ channel P domain residues. Numbered sites were studied by Pascual et al., (1995); those modified by external MTSET (arrows above) and internal MTSET (arrows below) are indicated. (i) Structure of a peptide toxin (α-KTx1.1, blue numbers) and 4 of its proposed *Shaker* K^+ channel contact sites (black numbers), after Naranjo and Miller (1996).

(MacKinnon, 1995). Pores are the catalytic active sites of ion channels. Pore structures, hotly pursued for clues to the mechanisms underlying ion permeation and selectivity, are now emerging (Sun et al., 1996; Lu and Miller, 1995; Pascual et al., 1995; Naranjo and Miller, 1996; Ranganathan et al., 1996; Gross and MacKinnon, 1996).

Inactivation Domains and Accessory Channel Subunits

Inactivation is a relatively voltage-insenstive step that either occurs over milliseconds and results from cytoplasmic channel domains acting as tethered open-channel pore blockers (Hoshi et al., 1990; Zhang et al., 1994;

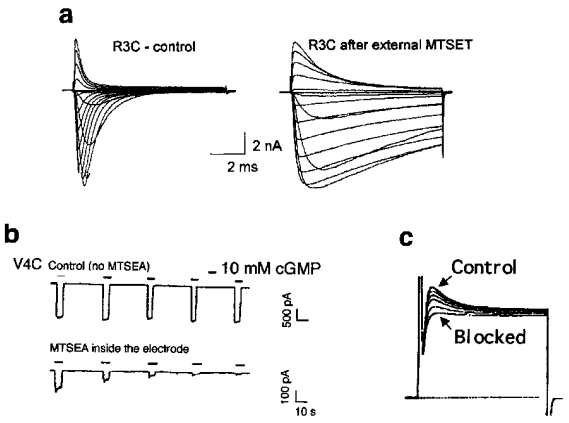

Figure 2. Channel Current Traces

(a) R3C Na^+ channels show altered inactivation after MTSET modification, from Yang et al. (1996). (b) V4C CNG channels are inhibited by MTSEA, from Sun et al. (1996). (c) Only the inactivating current is blocked by α-KTx1.1 when toxin-sensitive subunits are expressed with toxin-insensitive, noninactivating *Shaker* K^+ subunits at a ratio of 1:9, from Naranjo and Miller (1996).

Catterall, 1995), or is a slower process mediated by residues in S5, P, and S6 (Yellen et al., 1994). While the core (or α) subunits depicted in Figure 1 can function alone, they are expressed in most cells in association with one or more accessory subunits. These additional subunits are essential to integrated cellular function as they regulate channel expression levels and modify the functional activity of the core structures (Catterall, 1995).

S4 Charges Move in the Transmembrane Electric Field (Finally)

Na^+ Channels

Yang and colleagues (1996) investigate the S4 segment in the fourth homologous domain (D4) of a human skeletal muscle Na^+ channel. One at a time, they replace each of the eight positively charged S4/D4 residues with cysteine and use water-soluble, membrane-impermeant sulfhydryl reagents to determine exposure of each site to the external or internal aqueous solution. The highly lipophobic methanethiosulfonate (MTS) probes they use were introduced by Akabas et al. (1992) and, as seen in this review, now threaten to metastasize to all known membrane proteins. In earlier work, Yang and Horn (1995) found that a naturally occurring, disease-producing mutation of the first arginine in S4/D4 to cysteine (R1C, position 1448) created a channel that reacted with MTS-ethyltrimethylammonium (MTSET) in the external solution and that reaction was speeded by membrane depolarization. Recognizing this to be consistent with a voltage-dependent outward movement of the S4/D4 segment (and thus greater external exposure of R1C), they pursue the seven remaining basic positions.

Their results with R3C channels are particularly glorious. Like wild-type channels, depolarization induces a swift series of transitions in R3C channels from rest to open and then to inactivated conformation (equation 1). This is seen as a rapid rise and fall in Na^+ current (Figure 2a, control). Modification by MTSET dramatically slows inactivation of R3C channels (Figure 2a), while wild-type channels are unaffected. R3C channels are, however, only modified under special circumstances: external MTSET has no effect if the membrane is hyperpolarized (and channels are at rest) but slows inactivation upon depolarization. Conversely, internal MTSET has no effect if the membrane is depolarized but slows inactivation upon hyperpolarization. R3C thus appears to move completely across the membrane; exposed inside at rest, it gains exposure to the external solution with depolarization. In an enviable tour de force, the authors show that channels modified by external MTSET are protected by hyperpolarization from an external reducing agent but are unmodified when the reductant is added internally.

Proceeding in this fashion across the S4/D4 segment, Yang et al. (1996) offer responses to four topical questions. First, is S4 the voltage sensor? Yes, they argue. R1 moves partially and R2 and R3 completely across the membrane field. Each S4 segment can thus account for translocation of $\sim$2.5 elementary charges. Since the total charge movement per channel is approximately equal to that which underlies gating (Sigworth, 1994), they conclude that S4 is the primary voltage sensor. Moreover, modification proceeds as a first order voltage-dependent reaction with roughly the same kinetics as channel gating. Second, how "far" is it across the membrane? While R3C is accessible to internal MTSET at rest and external MTSET upon depolarization, R4C is accessible internally at both hyperpolarized and depolarized potentials. Since R3C can be extracellular when R4C is cytoplasmic, they argue that inside to outside can only be as far as the stretch from R3 to R4. Third, is gating charge movement the result of many charges moving short distances or do some charges move completely across the field? They argue forcefully for the latter. Fourth, why are so few charges present elsewhere in the membrane to stabilize S4? Counter charges are few, they suggest, because only one S4 charge is in the membrane at a time.

A hypothetical image of the structure of the channel based on these results has R1 partially hidden in an S4 canaliculus at rest (Figure 1d). Upon depolarization, S4 moves outward and R1, R2, and R3 enter a water-filled channel crevice that is in continuity with the external solution and large enough to allow MTSET ($\sim$6 Å) to enter (Figure 1e). R4 remains in the internal compartment, and the S4 canaliculus contains just two intervening hydrophobic residues. In α-helical conformation, the separation of R3 and R4 could be as little as 4.5 Å; if extended, no longer than 10–11 Å. As Yang and colleagues (1996) wisely note, how charge moves remains undetermined; it may be that the S4 backbone moves, or that the field moves around S4, or even that S4 is relatively stable but its arginine side chains alter position. In support of a model of S4 charge movement but only over a limited distance, French and colleagues (1996) show that a μ-conotoxin mutant sitting in the ion conduction pore shifts the voltage-dependence of activation as if repelling S4 charges electrostatically; they estimate that the center of S4 charge moves with depolarization, but only $\sim$5 Å. Yang et al. (1996) offer one last pearl. Saturating amounts of μ-conotoxin fail to protect R1C from external MTSET. This suggests R1

is outside the ~6 Å toxin footprint and that S4 canaliculi are distinct from the ion conduction pore.

K^+ Channels, Deja Vu?

Larsson and colleagues (1996) attack the S4 segment of a *Shaker* K^+ channel using the same strategy and make striking headway despite a painful constraint—positions that could not be studied. Homomeric assembly of four mutant subunits often ablates K^+ channel function when residues are critical. Undaunted, Larsson et al. exploit a rapid patch perfusion system to deliver MTSET to many sites across S4 in resting or open conformation and come to similar but not identical conclusions. First, they argue, yes, S4 charges move in the membrane field. R1C channels (position 362) are modified by external MTSET at rest, react more rapidly with depolarization, and never respond to internal reagent. R2C channels are modified by external MTSET only if depolarized and never respond to internal reagent, while R3C channels never respond to external MTSET and react with internal reagent only at rest. This suggests that with depolarization R1 and R2 move into continuity with the external solution, while R3 moves from the cytoplasmic solution to within three positions of the external solution. Although a full accounting of gating charge is not possible, they demonstrate persuasively that external exposure of R2 correlates in time and voltage-dependence with the outward flow of gating current (Manuzzu et al., 1996). Second, how far across? At rest, R1 is external, R2 is hidden, and R3 is internal; thus, at most, five residues span the membrane in closed channels. The boundaries of exposure are not yet defined in open channels (but may be wider). Third, gating charges may be more broadly distributed in K^+ channels. Fourth, this is consistent with interactions proposed for two acidic residues in S2 and S3 with S4 charges (Papazian et al., 1995).

Looking into the Pore from Inside and Out

cGMP-Gated Channels

The primary determinants of permeation and selectivity in voltage-gated Na^+, Ca^{2+}, and K^+ channels as well as in CNG channels reside in the P domain (MacKinnon, 1995). Sun and colleagues (1996) study this domain in a cGMP-gated channel from bovine retina using cysteine substitution and sulfhydryl probes. Reminiscent of problems seen with S4 mutants of *Shaker* K^+ channels, only 11 of 27 sites in the CNG P domain can be altered to cysteine and retain function as homotetramers. Nonetheless, marvelous insights accrue.

When exposed to internal cGMP, these ligand-gated channels move from resting to open conformation and pass cations (Figure 2b). cGMP appears to interact directly with cytoplasmic channel sites, and in its presence, channels remain open and do not inactivate (Goulding et al., 1994). MTS-ethylammonium (MTSEA) has no effect on wild-type channels when applied from outside or inside in the open or closed state. On the other hand, MTSEA blocks three channel mutants in the closed state from both sides of the membrane (Figure 1f; V4C, T20C, and P22C); this suggests that the barrier to ion flux in the closed conformation is just one residue thick! Two-sided access does not appear to result from occult channel openings. Rates of modification from both sides are similar in the absence of cGMP despite a spontaneous open probability of only $\sim 10^{-5}$. Access does appear to be by a water-filled pathway (and not obstructed by a selectivity filter), since T20C channels are also accessible from both sides to Ag^+, with a fixed positive charge, as well as to negatively charged MTS-ethylsulfonate (MTSES). That T16C and I17C are labeled only by internal MTSEA argues against the presence of a hydrophobic route. Sun et al. (1996) propose that CNG channel pores are at least as wide as MTSEA on both sides of an iris-like gate. They argue that the gate moves because V4C becomes inaccessible to MTSEA in the open state.

K^+ Channels Too?

Lu and Miller (1995) attack the P domain in *Shaker* K^+ channels using cysteine substitution but with two twists. First, they introduce the use of a K^+ analog, Ag^+, that reacts covalently with cysteine but is smaller in diameter (~2.5 Å) than MTS reagents and so more likely to move deeply into the pore. Second, they gain expression of 19 of 20 P domain cysteine mutants, many of which have no function as homotetramers, by studying channels with only a single mutant subunit. This is achieved by coexpressing cysteine mutants in a sea of subunits that have normal P domains but have their fast inactivation domain deleted; it is, thus, possible to study only those channels carrying at least one cysteine-labeled subunit by assessing the inactivating portion of the current (Figure 2c), a method introduced by MacKinnon et al. (1993). In the closed state, 11 P domain sites appear to be freely exposed to extracellular solution based on their rapid modification by Ag^+ (Figure 1g). Four contiguous sites that are unreactive, T9 (position 439), M10, T11, and T12, may reside on the inside of the closed channel with access to the internal solution, since T11 is known to mediate block by internal TEA (~8 Å) (Yellen et al., 1991). That T11 is separated by just one intervening residue from V13, a site exposed to external Ag^+, supports the notion of a short pore canaliculus.

Pascual and colleagues (1995) probe the P domain in a related voltage-gated K^+ channel (Kv2.1) and find evidence for a short pore canaliculus bounded by wide vestibules (Figure 1h). They observe that external MTSET travels as deeply as residue D17 (position 378), while internal MTSET modifies cysteines at positions T9, T11, T12, and V13. Thus, just three positions (G14, Y15, and G16) are inaccessible; notably, those sites hypothesized to be internal in *Shaker* K^+ channels prove to be accessible to internal MTSET in Kv2.1. Moreover, we are tantalized again by hints of two-sided access: while Ag^+ modifies *Shaker* V13 from the outside (Lu and Miller, 1995), MTSET modifies the analogous Kv2.1 site from the inside. Perhaps this should not be a surprise; CNG channel P domains share striking sequence and functional homology with K^+ channels (Heginbotham et al., 1992). This is underscored in a recently cloned cGMP-gated K^+ channel (Yao et al., 1995) and by the finding that CNG channels can be blocked from the inside by the N-terminal inactivation domain found in *Shaker* K^+ channels (Kramer et al., 1994).

Toxin Mapping of the K^+ Channel Pore

Naranjo and Miller (1996), Ranganathan and colleagues (1996), and Gross and MacKinnon (1996) bring the use

of peptide neurotoxins as probes of P domains to a new level of refinement. The α-K-toxins block K^+ channels from the external side (with picomolar affinity) by forming a 1:1 toxin–channel complex that occludes the pore. First used in an antediluvian search and destroy strategy that found toxin binding residues by mutation and thus led to the P domain (MacKinnon and Miller, 1989; MacKinnon, 1991), the toxins now provide an indirect means to assess spatial locations of pore residues. This is achieved by combining the known three-dimensional structure of the toxins and identified sites of toxin–channel interaction (see Miller, 1995; Aiyar et al., 1995). From a first crude image of the *Shaker* K^+ channel vestibule using charybdotoxin (α-KTx1.1) and a single weak steric contact pair (Goldstein et al., 1994), Naranjo and Miller (1996) now offer contact pair number six, M29 of α-KTx1.1 and *Shaker* T19 (position 449, Figure 1i), and the largest pairwise interaction energy yet observed (4.6 kcal/mol). The significance of this report lies in the fact that this contact was not stumbled upon; rather, it was predicted from the pair-driven model of the pore and thus validates its conclusions. A combinatorial method to analyze toxin binding to channels with nonequivalent subunits is introduced as well (Figure 2c).

Ranganathan and colleagues (1996) identify a long-sought channel contact partner. K27 is present in all α-K-toxins, it is critical to binding affinity and, because it interacts with K^+ ions traversing the *Shaker* pore (Goldstein and Miller, 1993), it is known to be in close proximity to the central axis of channel symmetry (Miller, 1995). K27 of α-KTx3.2 interacts with *Shaker* Y15 (position 445), a site in the "signature sequence" that mediates K^+ selectivity (Figure 1i) (see Heginbotham et al., 1994). This places the selectivity filter of the channel within just a few angstroms of the wide outer pore that holds the 15 × 25 Å body of the toxin and enjoys free contact with the extracellular solution. Gross and MacKinnon (1996) use lysine substitution and then a combined cysteine substitution and toxin protection strategy to make further exceptionally detailed predictions. They propose that the N-terminal side of the P domain loop approaches the central axis as an α helix (over part of its course); near the central axis, the loop dips below and then reemerges onto the underbelly of the toxin before progressing radially in an extended conformation.

Conclusions

A strong case can now be made that Na^+ channel S4 charges move short distances in the transmembrane electric field and that these charge movements can account for the voltage dependence of channel gating. S4 residues appear to traverse short canals (canaliculi) that link the internal and external solutions. This may serve to limit unfavorable interactions of gating charges and apolar protein and lipid domains. K^+ channel S4 charges are also on the move; they might be spread over more sites and employ somewhat longer S4 canaliculi.

An equally strong case can be made that the narrow parts of the ion conduction pathways in CNG and K^+ channels are short and, thus, that pores employ a similar energetic solution to moving charge across the membrane. Some properties of K^+ channels remain hard to reconcile with a short pore model (such as single-file occupancy by three or more K^+ ions and pore length estimates using bifunctional amine blockers; Villarroel et al., 1988), and information about residues forming the inner vestibule is still limited (but see Slesinger et al., 1993; Choi et al., 1993; Lopez et al., 1994). Nonetheless, a consistent hourglass image of the pore is developing: a wide outer vestibule that abruptly dives inward to form a short pore canaliculus and then opens again to a wide internal vestibule. It seems likely that inward rectifier (Kubo et al., 1993) and two P domain K^+ channels (Ketchum et al., 1995), which are equally selective for K^+ ions as their voltage-gated cousins, will have similar pore structures.

Caveats notwithstanding (is S4/D4 different than the other S4s; can MTS reagents squeeze into protein interstices; will protein movements or asymmetric subunit packing explain two-sided access), these eight papers are a high water mark for indirect evaluation of ion channel structure by site-directed mutation. They have confirmed some long-cherished ideas and offered intimations of physical details during this austere epoch preceding the crystallization and direct visualization of ion channel proteins.

Acknowledgments

I am grateful to Chris Miller and Fred Sigworth for reviewing this manuscript and to Amir Naini for thoughtful discussion and the image of CTX in Figure 1. S.A.N.G. is supported by the National Institutes of Health, the Donaghue Foundation, and a grant from American Home Products (Wyeth–Ayerst) to the Yale University Program in Molecular Cardiobiology.

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