Mini Review

Molecular Basis of Permeation in Voltage-Gated Ion Channels

Gordon F. Tomaseelli, Peter H. Backx, and Eduardo Marban

Ion channels are pore-forming macromolecules that provide a passive conduit for ion transfer across membranes. Although simple ionophores such as gramicidin and nystatin fall within this legalistic definition, the ion channels of eukaryotic cells are integral membrane proteins, many of which have now been isolated biochemically, reconstituted, cloned, and expressed. These fascinating proteins play a particularly prominent role in the function of the cardiovascular system by conferring excitability in an exquisitely tissue-specific manner and by mediating a number of key steps in signal transduction. The ion channels in a nodal cell enable it to pace, whereas those in a myocyte enable it to contract. In addition to mediating ionic fluxes across cell membranes, ion channels are the primary receptors for a variety of clinically important drugs including antiarrhythmic agents and calcium channel antagonists.

Therefore, it is of considerable interest to cardiovascular biologists to consider how ion channels work. Two distinct sets of properties characterize each type of ion channel: those that determine whether the channel is open or closed, collectively known as the “gating” properties, and those that define just how the channel moves ions when it is open, known as the “permeation” properties. The structure–function correlation of voltage-gated ion channels is the subject of several general reviews. The present Mini Review focuses specifically on the mechanism of permeation in ion channels, with particular emphasis on the structural domains that form the pore, determine selectivity, and interact with pore-blocking molecules.

Development of Molecular Approaches to the Study of Ion Channels

Cloning of the voltage-dependent Na⁺ channel from the electric organ of Electrophorus electricus in 1984 heralded a new era in electrophysiology. The availability of cDNA for ion channels enabled systematic analysis of structure by mutagenesis and heterologous expression. Since then, ion channels of many classes have been cloned, sequenced, and functionally expressed. (Ironically, the original eel Na⁺ channel has proven impossible to express by conventional methods.) Even before channels were expressed from cDNA, examination of the deduced amino acid sequences and hydropathy analysis demonstrated conservation of certain structural motifs. The voltage-gated ion channels consist of symmetrically arranged homologous subunits (or domains within a single large protein) surrounding a central pore. In the case of the Na⁺ channel α subunit, four homologous domains were predicted, each with at least six α-helical transmembrane repeats (S1–S6), as depicted schematically in Figure 1.1,2 Potassium channel proteins resemble a single domain of the Na⁺ channel1–3 and form the functional channel as tetramers.

The availability of cDNA clones enabled the marriage of the techniques of molecular biology and cellular electrophysiology, a particularly potent combination for studying the protein structure–function correlation. The general approach has been to select regions of the primary amino acid sequence, to alter the corresponding DNA sequences, and then to express the altered proteins in a system that can be studied biochemically and/or electrophysiologically. Phenotypic alterations in the mutant proteins may lead to clear-cut inferences about the channel structure.

The first task is the expression of functional channels in a heterologous system that permits their phenotypic characterization. Several expression systems are available: transfected mammalian cells, vaccinia virus–infected mammalian cells, baculovirus-infected insect intestinal cells, and oocytes of the clawed toad Xenopus laevis. The goal is to achieve high levels of protein expression for the ion channel of interest in cells that are devoid of similar endogenous channels and that are accessible to standard voltage-clamp or patch-clamp techniques for recording membrane currents. The next consideration involves choosing a region of the protein to change. Occasionally, clues come from evolutionarily conserved motifs with readily apparent functional implications; the fourth transmembrane repeat (S4) was correctly predicted, on the basis of its distinctive ribbon of positively charged residues, to sense transmembrane voltage and to initiate channel opening in response to depolarization.2 Natural divergence of amino acid sequences in similar channels is helpful in deciding which residue(s) might be functionally important; the pore region would be expected to be highly conserved evolutionarily in one given class of channels while differing substantially from the homologous region in channels with different selectivity.

491

From the Division of Cardiology, Department of Medicine, The Johns Hopkins University School of Medicine, Baltimore, Md.
Supported by National Institutes of Health grants RO1 HL-36957 (E.M.) and KO8 HL-02421 (G.F.T.) and the Medical Research Council of Canada (P.H.B.).
Address for correspondence: Eduardo Marban, MD, PhD, 844 Ross Building, The Johns Hopkins University School of Medicine, Baltimore, MD 21205.
Received August 24, 1992; accepted November 9, 1992.
Molecular Picture of the Pore From Shaker Potassium Channels and Their Homologues

Our understanding of K⁺ channel structure and function made a major advance with the cloning of the Shaker locus in Drosophila melanogaster. This locus encodes a family of alternatively spliced transcripts that code for transient or A-type K⁺ channels. Three other subfamilies of voltage-dependent K⁺ channels, Shal, Shab, and Shaw, have been cloned from Drosophila, and homologous mammalian genes have been described for each subfamily. The core or membrane-spanning regions (S1–S6) of the K⁺ channels from each subfamily share approximately 40% amino acid identity, whereas the amino and carboxy termini tend to be quite variable.

A region of particularly marked sequence conservation occurs between the fifth and sixth membrane-spanning regions (S5–S6), alternatively known as H5, SS1–SS2, or the P loop (Figure 2). The first clue that this loop, which was first proposed to be extracellular, in fact reenters the membrane and forms the pore came from experiments probing the interaction of a polypeptide blocker from scorpion venom, charybdotoxin, and the Shaker K⁺ channel. Mutagenesis revealed at least two mechanistically distinct classes of sites: One set of residues (including positions 5 and 10; see Figure 2 for numbering convention) influences block by altering toxin binding in a manner that can be easily rationalized by the local change in potential produced by altering the charge of the residue at these positions. On the other hand, changes at position 14 (aspartate in wild type), 32 (threonine), and 33 (valine) dramatically affect block by charybdotoxin but not in a way that is explicable by a change in local charge. Because charybdotoxin blocks the pore when it binds, these experiments support the hypothesis that the S5–S6 region of the channel contributes to the formation of the ion conduction pathway.

Although the experiments with charybdotoxin pointed to the general area that mediates permeation, this toxin is a large molecule (≈2.5×1 nm) and likely interacts with residues outside the pore itself. A smaller blocker, tetraphenylammonium (TEA), blocks K⁺ channels from the extracellular surface with variable affinity depending on the channel subtype. Yellen et al. found two classes of residues that influenced external TEA blockade of the Shaker channel. Changing the residue at position 14 increased the EC50 monotonically with increasing charge, consistent with an electrostatic mechanism. Changing other residues, glutamic acid–5 and lysine–10, that influenced charybdotoxin binding had no effect on external TEA blockade. In contrast, the sensitivity of this K⁺ channel to TEA depended critically on the residue at position 32. In the wild-type channel, there is a threonine at this position; changing it to a lysine, the corresponding residue in the TEA-insensitive mammalian homologue RCK4, eliminated the external TEA sensitivity of the channel. Substitution of either valine or glutamine at this position also reduced or eliminated TEA sensitivity. On the other hand, substitution of an aromatic residue, tyrosine, known to exist at this position in the mammalian K⁺ channel homologues drk1 and RCK1, increases the sensitivity of the channel to external TEA over 50-fold. The mutations with lysine, arginine, or glutamine at position 32 demonstrated reduced single-channel current amplitude and loss of the normal inward rectification, leading the authors to conclude that the changes produced by the mutations involved specific alterations in the conduction pathway.

Further support for the role of this region as part of the pore of voltage-gated K⁺ channels emerged from several groups. Yellen et al. again using TEA as a probe but this time from the cytoplasmic face of the Shaker K⁺ channel, defined the importance of the residue at position 24 in determining the sensitivity to TEA from the inside. Changing this residue to a serine increased the sensitivity to internally applied TEA.
fourfold without affecting the sensitivity to externally applied blocker. TEA blocked the channel in a shallow voltage-dependent manner equivalent to approximately 15% of the total electrical field when applied from the inside and in a nearly voltage-dependent manner when applied from the outside. The implication is that from residue 24 to 32 nearly 85% of the membrane field is traversed. An $\alpha$ helix of this length would span only 1.2 nm; alternatively, the S5–S6 loop could assume a more extended conformation, such as a $\beta$ strand, which would span $\approx2.7$ nm. In either case, the pore of the channel appears to be much shorter than the width of the bilayer, as originally suggested from classical experiments on native K$^+$ channels.1

Yool and Schwarz2 examined the effect of mutations in the S5–S6 region on the selectivity of Shaker channels. Mutations at positions 16 and 24 produce channels with altered ionic selectivity. A decrease in the bulk and hydrophobicity of the side chains of the residues at these positions increases the permeability of NH$_4^+$ compared with the wild-type channel without substantially affecting the K$^+$ conductance. These mutations, F16S and T24S, produce channels with significantly larger inward conductances to both NH$_4^+$ and Rb$^+$ than wild-type Shaker channels, but retain the ability to exclude Na$^+$.7

Hartmann et al8 constructed chimeras of two mammalian K$^+$ channels, drk1 and NGK2. A stretch of 24 amino acids from NGK2 (residues 20–39 of the P loop and the first four residues of S6, Figure 2) was substituted for the analogous sequence of the drk1 channel. NGK2 has a threefold higher single-channel conductance, 20-fold higher affinity to external TEA, and 100-fold lower affinity to internal TEA compared with drk1. The chimera has a phenotype resembling that of the donor NGK2 channel: a larger single-channel conductance, high sensitivity to external TEA, and low sensitivity to internal TEA. Thus, this stretch of 24 amino acids contains all the information necessary to determine the pore phenotype of these mammalian K$^+$ channels.8 Extension of this work using point reversions of residues in the NGK2 region of the chimeric channel suggested that the deep region of the pore was between proline 13 and proline 33. Further, nonpolar residues at positions 21 and 26 are important in determining the K$^+$ conductance, providing evidence that the pore is formed not by charged or polar side chains but by the backbone of a hydrogen-bonded $\beta$ sheet composed of nonpolar residues.8 Comparable chimera and site-directed mutagenesis experiments on other mammalian K$^+$ channels support the previous findings.

Figure 2, bottom panel, summarizes the mutagenesis results for the S5–S6 loop of K$^+$ channels. The picture that emerges is of a short stretch of residues between the fifth and sixth membrane repeats that turns back into the membrane and creates the pore. A predicted $\beta$ hairpin may be contributed from each of four subunits to form a $\beta$-barrel structure, although the precise conformation is not yet known. It is notable that a similar structure for the Na$^+$ channel pore was predicted by
Guy and Seetharamulu\(^{10}\) well before the cloning of the first Shaker K\(^+\) channel.

The Sodium Channel Pore: Analogs to Potassium Channels

In considering the structural basis of permeation in Na\(^+\) channels, it is natural to focus on S5–S6. Although that segment of the Na\(^+\) channel exhibits little sequence homology with the S5–S6 of Shaker or mammalian K\(^+\) channels, such divergence is not surprising given that the pore structure must differ fundamentally in these two channel families. In contrast, this region is highly conserved among Na\(^+\) channels in all four domains (Figure 3), corroborating its functional significance.\(^{10}\)

There also turned out to be many differences (but some tantalizing homologies, discussed below) between Na\(^+\) and Ca\(^{2+}\) channels in the S5–S6 region (Figure 3). Again, conservation within the Ca\(^{2+}\) channel family was striking.\(^{1}\)

In much the same way that charybdotoxin binding relates to ion permeation in the K\(^+\) channel, tetrodotoxin (TTX) and saxitoxin (STX) are Na\(^+\) channel blockers whose binding sites almost certainly involve the pore. Experiments using radiolabeled-toxin binding and electrical recording in nerve have implicated an acidic site in TTX/STX binding and ion flux through the channel. Evidence for the proximity of the TTX binding site to the pore of the Na\(^+\) channel comes from studies of biochemical modification of the channel protein. Trimethyloxonium tetrafluoroborate, a reactive reagent that esterifies carboxyl oxygens, reduces single-channel conductance, sensitivity to TTX/STX, and blockade by externally applied Ca\(^{2+}\). Chemical modification of the channel by trimethyloxonium tetrafluoroborate can be prevented by pretreatment with TTX or Ca\(^{2+}\), consistent with the notion that methylation of a single specific carbonyl group mediates this effect.\(^{1}\)

By using the structural analogy to the K\(^+\) channel and the known importance of an acidic group near the pore for toxin binding, site-directed mutagenesis of the S5–S6 segment was initiated on a rational basis. The collaborative efforts of the laboratories of Numa and Stühmer (Terlau et al\(^{11}\)) provided the sentinel work. Neutralization of a single glutamic acid residue in the P loop of domain I eliminated the toxin sensitivity of a TTX-sensitive Na\(^+\) channel from rat brain expressed in Xenopus oocytes. The small unitary conductance and reduced toxin sensitivity of this mutation (I-E54Q; see Figure 3 legend for numbering convention) resemble channels O-methylated by trimethyloxonium tetrafluoroborate, suggesting that this residue may contain the carbonyl that is modified when trimethyloxonium tetrafluoroborate is applied to the outside of the channel. Comparison of the sequences of TTX-sensitive (e.g., brain and skeletal muscle) and TTX-resistant (e.g., heart) channels in this region reveals conservation of the glutamic acid residue at this position, but in the cardiac channel the adjacent residue is an arginine (I-R55) rather than an asparagine (Figure 3). This substitution was thought to have the functional effect of neutralizing the local charge produced by glutamate 54, but it is now clear that other residues play a dominant role in determining toxin sensitivity.

Terlau et al\(^{11}\) found mutations in the putative P loops of all the domains that influence toxin binding, most of which also reduced single-channel conductance (Figure 3). Complementary experiments using chimeric constructs of TTX-sensitive and TTX-insensitive channels suggest that the primary determinant of toxin sensitivity is in domain I in a 22-amino-acid stretch near the carboxy terminal end of the S5–S6 linker.\(^{12}\) Examina-
tion of the sequences in this region reveals several differences, only one of which involves a charged residue (Figure 3). It is apparent that the toxin binding site is complicated and involves more than an electrostatic interaction between rings of charge on the Na⁺ channel and the positively charged toxin. Schidl and Moczylowski used the sulhydryl-specific alkylating agent iodoacetamide to modify mammalian cardiac Na⁺ channels in planar lipid bilayers and demonstrated a 20-fold reduction in STX sensitivity in the modified channels. This modification also eliminated high-affinity blockade of the channels by Zn²⁺. These results suggested that the known sensitivity of cardiac Na⁺ channels to group IIB metals and insensitivity to TTX/STX may be mediated by a critically positioned cysteine residue in the pore.

Using the rat heart channel as background, Satin et al. mutated a cysteine to tyrosine (I-C52Y) and produced a Na⁺ channel with markedly increased sensitivity to TTX. Backx et al. made the complementary mutation in the skeletal muscle Na⁺ channel, converting tyrosine to cysteine (I-Y52C) and thereby rendering the channel TTX resistant. Single-channel recordings revealed that the mutant channels are three orders of magnitude more sensitive to block by Cd²⁺ and Zn²⁺ than are the wild-type skeletal muscle channels. Block by divalent cations was voltage dependent in both variants, and the fractional electrical distance equaled approximately 20% (from the outside) in both cases.

Comparison of the sequences of the putative P-loop regions in all four domains of the Na⁺ channel with those in the α subunit of the Ca²⁺ channel (Figure 3) reveals important differences at the positions that determine toxin sensitivity in the Na⁺ channel. The role of these residues in ion selectivity was determined by site-directed mutagenesis to glutamate, the residue that is found in the comparable position in Ca²⁺ channels. Using bi-ionic reversal potential experiments, the selectivity of these mutants was determined and compared with the wild type. Both mutants III-K26E and IV-A33E demonstrated reduced selectivity for Na⁺; particularly interesting was the susceptibility of these mutants to block by micromolar concentrations of externally applied divalent cations. When divalent cations are depleted, native Ca²⁺ channels also support high monovalent cation fluxes; trace amounts of divalent cations block monovalent current, but higher concentrations support divalent permeation. Such anomalous mole-fraction behavior represents the strongest evidence for multi-ion occupancy of the Ca²⁺ channel pore. The mutation III-K26E behaves much like voltage-dependent Ca²⁺ channels, showing reduced Na⁺ conductance in the presence of Ca²⁺ but being permeable to both Ca²⁺ and Ba²⁺ at millimolar concentrations. Remarkably, the double mutant III-K26E·IV-A33E is not only permeable to Ca²⁺ but is actually selective for Ca²⁺ over Na⁺ at physiological concentrations. Thus, the essential features of permeation in Ca²⁺ channels have been recreated by point mutations in the Na⁺ channel gene without changing other features of the channel such as gating kinetics. Thus, as in K⁺ channels, the S5–S6 region in each domain of the Na⁺ channel is pivotal in determining the ion selectivity of the pore.

Studies on Calcium Channel Permeation Are Nascent

There are at least four biophysically and pharmacologically distinct subtypes of Ca²⁺ channels, P, T, N, and L. The L-type calcium channel, also referred to as the dihydropyridine (DHP) receptor for its ability to bind drugs of the nifedipine family, is the most important in the cardiovascular system. Photoaffinity labeling of purified DHP receptors from skeletal and cardiac muscle shows that DHP ligands are incorporated primarily into a single polypeptide with a molecular mass of 170 kD, the α subunit. This subunit has been cloned, sequenced, and expressed from several tissue sources and contains most if not all of the protein's functional domains.

The anomalous interactions among monovalent and divalent cations discussed above suggest that permeation in Ca²⁺ channels involves simultaneous occupancy of the pore by multiple ions, with each ion binding to a specialized high-affinity site. The high binding affinity explains the exquisite selectivity of Ca²⁺ channels, whereas simultaneous occupancy maintains high flux because of electrostatic ion–ion repulsion. It would be of great interest to define the nature of these sites. Unfortunately, expression of functional Ca²⁺ channels in conventional systems is not as robust as it is with other voltage-dependent channels. Particularly useful in this regard were skeletal muscle cells from dysgenic mice that lack DHP-sensitive Ca²⁺ current and excitation–contraction coupling. Injection of skeletal muscle or cardiac Ca²⁺ channel α-cDNA into the nuclei of cultured myotubes from dysgenic mice reconstituted both the DHP-sensitive Ca²⁺ current and excitation–contraction coupling in these cells, supporting the dual role of the DHP receptor as a Ca²⁺ channel and voltage sensor for excitation–contraction coupling. Nevertheless, this is a technically difficult expression system that is not readily amenable to single-channel recordings (because of the presumed concentration of the expressed Ca²⁺ channels within transverse tubules). With the cloning and coexpression of minor subunits and the utilization of other systems, improved functional expression has been realized; this should facilitate the molecular dissection of the pore.

Much of the information regarding the permeation pathway of the Ca²⁺ channel is inferential and comes from comparison of the sequence of the α subunit with the α subunit of the Na⁺ channel and the K⁺ channel, as reviewed above. The other major source of information is affinity labeling studies of the α subunit from skeletal muscle. The DHP and phenylalkylamine binding regions of the α subunit have been localized using photoaffinity probes. These agents are open-channel blockers; thus, their binding sites are presumed to be near the pore. Electrophysiological studies using charged membrane-impermeant DHP and phenylalkylamine derivatives suggest that they block from the outside and inside, respectively. Using the phenylalkylamine [N-methyl–H]LU49888, Striessnig et al. have localized the binding site of this class of antagonists to the fourth domain of the subunit in a highly conserved region, the carboxy terminal to the sixth transmembrane segment (S6). If the α subunit of the Na⁺ channel and the α subunit of the Ca²⁺ channel are
topologically comparable, this would place the binding site near the cytoplasmic mouth of the channel.\textsuperscript{10} Similar studies using DHP affinity probes have provided variable results placing the DHP binding site in the region of S6 either in the third or fourth domain. In either case, labeling occurs in conserved regions of the peptide that are predicted to be near the outer entry-way to the pore.\textsuperscript{13}

In summary, these data suggest that, as for the Na\textsuperscript{+} channel, the pore of the Ca\textsuperscript{2+} channel is formed by contributions from several of the internally conserved domains. A loop between S5 and S6, as has been proposed for the other voltage-gated ion channels, is not inconsistent with the observations to date.

Conclusions and Future Directions

Significant progress has been made in understanding the molecular basis of permeation in voltage-gated ion channels, but substantial gaps in our knowledge remain. The use of recombinant DNA techniques in combination with high-resolution electrical recording has provided new insights at a staggering pace. Since the region of the primary amino acid sequence that forms the pore in the intact channel protein has been defined, questions regarding the processes of transport and selectivity can now proceed to a new, more mechanistic plane. Of particular importance to cardiovascular science is the tantalizing possibility that rational design of channel subtype-specific ligands may become realizable as our understanding of the permeation process becomes more refined.

References

Note: The number of references in Mini Reviews is restricted by editorial policy, but a complete bibliography will be provided by the authors upon request.


10. Guy HR, Seetharamulu P: Molecular model of the action potential sodium channel. Proc Natl Acad Sci USA 1986;83:508-512


13. Schild L, Moczylowski E: Competitive binding interaction between Zn\textsuperscript{2+} and saxitoxin in cardiac Na\textsuperscript{+} channels: Evidence for a sulphhydryl group in the Zn\textsuperscript{2+}/saxitoxin binding site. Biophys J 1991;59:523-537


17. Striessnig J, Murphy BJ, Catterall WA: Dihydropyridine receptor of L-type Ca\textsuperscript{2+} channels: Identification of binding domains for [\textsuperscript{3}H](+)-PN200-110 and [\textsuperscript{3}H]azididine within the \(\alpha\) subunit. Proc Natl Acad Sci USA 1991;88:10769-10773

18. Striessnig J, Glossmann H, Catterall WA: Identification of a phenylalkylamine binding region within the \(\alpha\) subunit of skeletal Ca\textsuperscript{2+} channels. Proc Natl Acad Sci USA 1990;87:9108-9112


Molecular basis of permeation in voltage-gated ion channels.
G F Tomaselli, P H Backx and E Marban

Circ Res. 1993;72:491-496
doi: 10.1161/01.RES.72.3.491

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/72/3/491.citation