The Structure of Ion Channels in Membranes of Excitable Cells

Review

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Ion channels play a central role in the transmission of electrical signals along the membranes of neurons and other excitable cells; they also mediate communication between cells at synapses and participate in a multitude of regulatory processes involving signal transduction at the membrane surface. For a single molecule these represent tasks that are both diverse and complex. Yet, on the face of it, channels are remarkably uncomplicated. First, they appear to share the same, simple architectural plan. The building blocks are identical or homologous polypeptide units, which are assembled symmetrically within the membrane (at least where structural evidence is available), delineating a pore down their center. Second, channels have just two essential states—open or closed. Either the pore is sealed, making a permeability barrier across the membrane, or it forms a continuous, water-filled pathway, exposing the surfaces needed to discriminate between the ions passing through.

This is the elementary picture. At a molecular level, the detailed structure of channels has not been resolved, and so fundamental questions remain unanswered. What is the design that makes channels so efficient, facilitating selective transport across the membrane typically of thousands of ions in a millisecond? What are the chemical and physical origins of ion transport specificity that allow channels to hinder or prevent diffusion of some ions but not of others across the membrane? What conformational changes are involved in gating—the rapid switching between open and closed states? Do channels make use of the same general rules that apply to soluble enzymes or multisubunit complexes, or does the presence of the lipid bilayer enforce something basically different?

Of course, a really sound understanding will have to await the three-dimensional framework furnished by structures solved crystallographically to atomic resolution. In the meantime, however, channels are becoming much better understood physiologically and biochemically as a result of powerful new techniques such as patch-clamping, molecular cloning, and site-directed mutagenesis, which enable single-channel behavior to be recorded, amino acid sequences to be derived, and specific parts of the sequence to be related to precise physiological functions. New channel proteins are being discovered and characterized. Superfamilies of voltage- and ligand-gated channels are being identified and extended. And experiments are now even pin-pointing certain amino acid residues as lining the walls of pores, as affecting rate of ion transport, and as involved in sensing voltage shifts across the membrane.

The purpose of this review is to discuss implications of some of the recent findings in relation to three-dimensional structure, drawing on information derived from studies of channels, other bilayer-spanning proteins, and soluble proteins by electron microscopy and X-ray diffraction. It is likely that channels have parallels with soluble allosteric enzymes. The fact that both types of molecules switch states reversibly in an all-or-none fashion, the cooperative behavior of many channels, and the conformational changes detected in channel proteins at low resolution all suggest the existence of close structural analogies. Obviously, the lipid bilayer is also important, and must restrict the conformational changes that would be energetically acceptable, since it is a separate phase that the protein surfaces are constrained to match.

Ion channels, like soluble proteins, often take part in a range of actions. Not only do they detect and transmit the effect of ligand binding or shifts in membrane potential, but they interact with various cytoplasmic and extracellular molecules as well. These extra interactions may involve intimate associations with other proteins or necessitate separate structural specializations of the channel protein itself. Such individual properties and the classification of channels according to their gating characteristics, or to the superfamilies in which they belong, have been discussed in several recent reviews (e.g., Hille, 1984; Catterall, 1988; Miller, 1989). The emphasis here is on features that channels may have in common. Since these molecules have all evolved with the same task of mediating passive transport across the membrane, it is possible that they are constructed and work by a similar set of underlying rules, whatever the differences in subunit composition, in the fine detail, or in the specializations they incorporate.

In keeping with a unifying theme, I shall show that putative pore-lining α-helices of channels in different superfamilies share identical alignments of small polar and large hydrophobic residues. This implies that there is a common packing principle involved in building the wall around a pore and suggests a possible general way by which channels may open and close.

Channels as Oligomeric Proteins

Channels are essentially oligomeric membrane proteins possessing cyclic or pseudo-cyclic symmetry (Figure 1), and one way to classify them is in terms of the number of subunits, or structural units, around the ring. Although in some cases this number has been established directly by experiment, in others it has been deduced indirectly from the amino acid sequence. Either there are homologous bilayer-spanning domains, representing the structural units, within the sequence of a single polypeptide (in which case it is not strictly an oligomer), or the protein shows homologies with a channel protein in which the number of structural units has already been
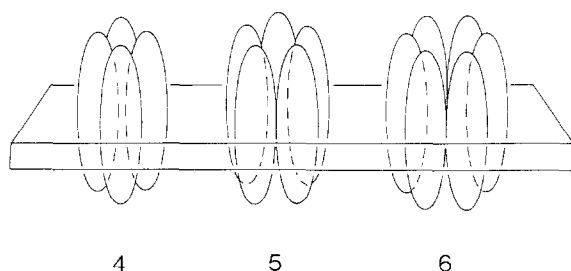
4   5   6

Figure 1. Ion Channels Are Built from Four, Five, or Six Structural Units

The channels discussed in this review are all thought to be integral membrane proteins possessing cyclic or pseudo-cyclic symmetry, in which the central symmetry axis delineates a gated, water-filled pathway for the ions. The structural units are identical or homologous protein subunits, or homologous domains comprising a single polypeptide chain. The most selective channels, with the narrowest pores (e.g., the voltage-gated sodium and calcium channels), have four structural units; the least selective channels, with the widest pores (e.g., the gap junction channel), have six units; and the channels with intermediate properties (e.g., the nicotinic acetylcholine receptor) have five units.

determined. The tertiary folding and overall three-dimensional form are the same in proteins whose polypeptide chains are homologous over their length, and so inferences from homologies concerning oligomeric state are probably correct.

There are now several well-studied examples of channels in the four, five, and six structural unit class. The voltage-gated sodium channel and calcium channel (dihydropyridine receptor) are both composed of four homologous bilayer-embedded domains, making up a large fraction of a single polypeptide chain, and so presumably consist of four structural units in a ring (Noda et al., 1984; Tanabe et al., 1987). The same may be true for the voltage-gated A-type potassium channels, whose polypeptides have some homologies with these domains (Tempel et al., 1987; Timpe et al., 1988). And fourfold symmetry of the ryanodine receptor (a calcium channel in sarcoplasmic reticulum) has been demonstrated by electron microscopy (Lai et al., 1988; Wagenknecht et al., 1989).

A five-subunit stoichiometry of the muscle nicotinic acetylcholine receptor was found originally by preparative gel electrophoresis (Lindstrom et al., 1979) and by quantitative amino-terminal analysis (Raftery et al., 1980). Other neurotransmitter-gated receptors, such as the neuronal acetylcholine receptor, the γ-aminobutyric acid ($GABA_A$) receptor, and the glycine receptor, are composed of polypeptides that are homologous with those of the muscle nicotinic acetylcholine receptor over almost their entire length (Schofield et al., 1987; Grenningloh et al., 1987) and would therefore be expected also to have five subunits. Chemical cross-linking experiments (Langosch et al., 1988) have provided additional support for a pentameric structure of the glycine receptor.

Yet other channels with wider pores, such as the gap

junction channel and synaptophysin, have been determined by structural methods (Makowski et al., 1977; Unwin and Zampighi, 1980) or cross-linking studies (Thomas et al., 1988) to be hexamers of identical subunits.

Quaternary Structures

None of the above channels has yet been analyzed at anywhere near atomic resolution, and some we know about only through single-channel conductance measurements and deduced amino acid sequences. However, it has been possible to discover details about two of these channels at low resolution by three-dimensional electron image analysis of molecules crystallized in membranes as planar or helical arrays. The best images have been obtained by rapidly freezing the specimens in a thin film of solution and observing the specimens at a temperature low enough ($< -130°C$) for the ice to remain stable. A defined lipid and ionic environment is captured by the freezing, providing closer ties with the physiology than had been feasible previously using negative stains to preserve shape and provide contrast. Furthermore, the protein is seen directly, contrasted against the lipid and water molecules, making it possible to detect and analyze small conformational changes. What are the significant features in three-dimensional maps from the cryo-images?

First, some background on the gap junction channel—one of the channels studied in this way. This channel is built from a ring of six identical, ~75 Å long subunits, called connexins. Several homologous polypeptides belonging to the connexin family have been discovered, the best characterized of which are the 32 kd and 43 kd polypeptides from, respectively, liver and heart (Henderson et al., 1979; Paul, 1986; Kumar and Gilula, 1986; Beyer et al., 1987; Young et al., 1987). Connexins are unusual because of their dual role: they associate together within the plasma membrane of one cell to make the channel, and they link to connexins in the plasma membrane of another cell to make a continuous communicating pathway between the two interiors (Loewenstein, 1981). The central pathway, when open, is wide (~16 Å minimum diameter; Schwarzmann et al., 1981) and relatively nonspecific, allowing the passage of signal molecules such as cAMP, as well as ions, between connected cells. Calcium and hydrogen ions are ligands that regulate the transport, but the channel closes, rather than opens, in response to raised cytosolic calcium levels or lowered pH (Rose and Loewenstein, 1975; Noma and Tsuboi, 1987). The transition is cooperative, and Hill coefficients of 3–4.5 have been estimated (Spray et al., 1982). Desensitization does not occur, and thus different ion concentrations can be used, in principle, to stabilize either of the two states.

Since the gating action involves large changes in pore dimensions, the accompanying conformational changes should be large and hence detectable in the cryo-images. Results obtained from the channels in isolated gap junctions appear to bear this out (Unwin and Ennis, 1983, 1984). In calcium-free solution, the rod-shaped subunits
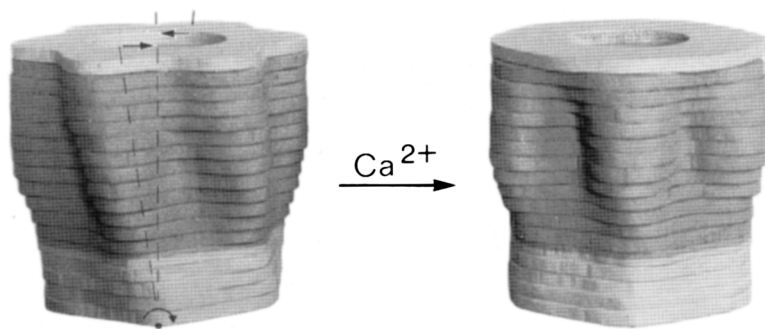
Figure 2. Two Configurations of an Ion Channel

Two calcium-sensitive configurations of the gap junction channel have been revealed by cryo-electron microscopy and three-dimensional image analysis. Subunits on either side of the pore tilt in opposite directions on exposure to calcium, producing tangential displacements (pair of arrows) that are greatest at the upper, cytoplasmic end of the structure. The subunits move inward and narrow the pore near the cytoplasmic membrane surface as a result of this action. Taken from Unwin and Ennis (1984).

are tilted by 7°–8° around the central axis delineating the pore (Figure 2, left); when calcium is added they become aligned approximately parallel to this axis, and the pore, near the cytoplasmic membrane surface, becomes more constricted (Figure 2, right). Moreover, the shape of the subunits is not affected by the calcium. Hence the switch in conformations is a simple rearrangement: tilting of the subunits about axes at one end of the structure is linked to tangential displacements of the subunits (of up to ~9 Å) at the other, leading to changes in the diameter of the pore.

Is this structural transition the one responsible for the opening/closure event detected by physiological experiments? This has yet to be demonstrated. However, a high degree of cooperativity is implied, since all of the subunits must change their mode of packing. Furthermore, the rearrangement could move points on the pore-lining surfaces of the subunits toward or away from the central axis over a distance comparable to their tangential displacements (i.e., up to 9 Å). Thus, although the amino acid side chains involved have not been resolved and these determine the precise dimensional changes, the tilting mechanism would appear to provide enough structural modification to account for the physiological results.

Soluble, multisubunit enzymes and more extended assemblies such as viruses undergo conformational switches involving concerted rearrangements of essentially rigid units, which are similar in both magnitude and character to these just described. The binding of hydrogen ions, divalent cations, or RNA to the disc form of tobacco mosaic virus protein triggers its conversion to the helical form, as in the virus, by changes in tilt and displacement of its rod-shaped subunits (Klug, 1972). The two αβ dimers composing the hemoglobin tetramer each rotate 6.5° in opposite directions upon oxygenation and in so doing reduce the width of a cavity in the center of the tetramer by 2–3 Å (Perutz, 1989). The central cavity lies perpendicular to the rotation axis, and the channel runs perpendicular to the tilt axis. So the deoxy–oxy conformational change appears to provide a close analog of the channel transition.

The nicotinic acetylcholine receptor is the other channel whose quaternary structure has been analyzed by the cryo-methods. This cation-selective channel has an open pore smaller (7 Å diameter; Dwyer et al., 1980) than that of the gap junction channel and is the more sophisticated of the two, being finely tuned to respond briefly to the release of acetylcholine and to inactivate (or desensitize) if the application is prolonged. It is constructed from similar rod-shaped subunits (α2, β, γ, and δ) around a pseudo five-fold axis (Changeux et al., 1984; Karlin et al., 1986; McCarthy et al., 1986), rather than a six-fold axis. The subunits are oriented approximately normal to the membrane plane and protrude far into the water, especially on the side facing the synaptic cleft. They are arranged, according to crystallographic labeling experiments (Kubalek et al., 1987), with the β subunit between the two α subunits and with the γ and δ next to each other on the opposite side (Figure 3). Ho-
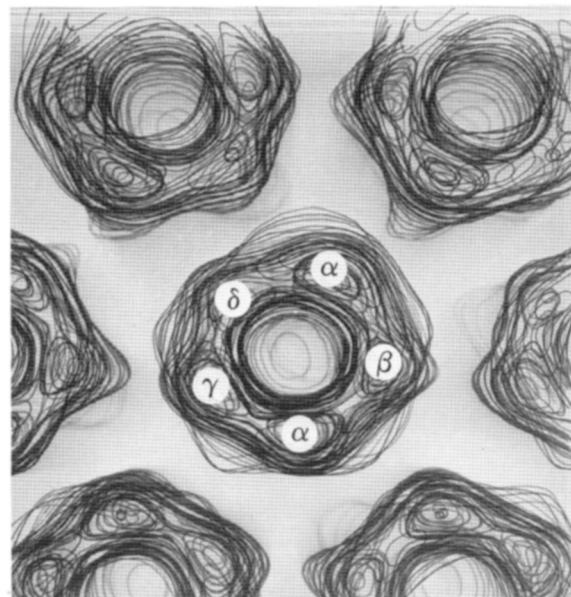

Figure 3. Pentameric Structure of the Nicotinic Acetylcholine Receptor

Three-dimensional contour maps of this receptor have been obtained by crystallographic analysis of cryo-images from Torpedo postsynaptic membranes. The long, ~25 Å diameter entrance to the channel and encircling five subunits are as they would appear viewed from the outside of the cell. Adapted from Brisson and Unwin (1985).

mology between the subunits (~40% identity in amino acid sequence, on average, comparing one chain with any of the others; Noda et al., 1983b) means that they all incorporate the same tertiary folds and structural motifs. Subunit–subunit interactions could therefore be similar around the ring. So, do these subunits—like the gap junction subunits—participate equally to achieve a different functional state?

Experiments have suggested otherwise. For example, hybrid calf/Torpedo acetylcholine receptors, expressed in oocytes, show widely different gating behavior depending on which combination of subunits is assembled (Sakmann et al., 1985); remarkably, the Torpedo receptor having only its δ subunit substituted with the calf δ subunit gives single-channel open times similar to those of the calf receptor, rather than to the parental Torpedo molecule. Different structural responses of the individual subunits to agonist have been seen by comparing three-dimensional maps from cryo-images of the Torpedo receptors with and without agonist present (Unwin et al., 1988). When the acetylcholine analog carbamylcholine is in the buffer, under conditions that would promote desensitization, the δ subunit becomes more inclined tangentially around the channel and the γ subunit is displaced slightly outward; the remaining subunits are less noticeably affected.

So the asymmetry is there for a purpose. One possibility is that by distorting unevenly the configuration of subunits around the pore, the quaternary change leading to desensitization stabilizes, or locks, the closure. It will be interesting to see whether switching to the open state, induced by cooperative binding of acetylcholine to the two α subunits, involves more equal participation of the encircling subunits. The allosteric enzyme, phosphofructokinase (a tetramer of identical subunits), switches from one symmetrical configuration to another in the transition from the T-state to the R-state (Schirmer and Evans, 1990), whereas hemaglobin switches from a more to a less symmetrical configuration (Perutz, 1989). Both kinds of quaternary change could be facilitated by a structure composed of similar, but nonidentical, subunits.

Tilting across the Membrane

These low resolution structural studies perhaps hint that channels may generally utilize small tilting rearrangements of some or all of their subunits to switch between open, closed, and inactivated states: actions that could be triggered by localized conformational changes due to ligand binding or be coupled with charge movements produced by a shift in membrane potential. Is it reasonable to suppose this, or might there be too much cost in free energy? In fact, the energy costs need not be great. A large local effect—such as the opening of a pore—can be produced by the concerted tilting of several subunits without the energetically unfavored processes of creating substantial new surfaces or invoking gross distortions of the subunits themselves. Furthermore, the movements entailed are predominantly paral-

lel to the membrane, rather than away from it, so that the partitioning of the polar and nonpolar protein surfaces between water and lipid remains essentially unperturbed.

Other experimental observations also fit the view that significant rearrangements of this sort could be widely utilized. The activation of voltage-gated sodium channels shows a strong pressure dependence, indicating that a considerable portion of this molecule participates in the conformational switch to open the channel (Conti et al., 1984). The fact that fully functional hybrid acetylcholine receptor channels can be produced by substituting original subunits with homologous ones of the same family (Sakmann et al., 1985) and even of a different, but related family (Deneris et al., 1988) suggests that the subunit–subunit interfaces do not entail extensive or tight, interdigitating contacts as would make the rearrangements difficult. Hemaglutinin is an example of an oligomer that protrudes from the membrane like the walls of a channel, and the subunit–subunit contacts, except for a central stabilizing portion, are not extensive (Wilson et al., 1981).

It is tempting to speculate that relative displacement of one subunit surface against another by tilting (i.e., rotation about an axis parallel to the membrane plane) may be a key component of many processes involving communication across the membrane. The effect of ligand binding on one side can be transmitted to the other side and even amplified by that means. A net movement of charge mediated by switching of interactions between amino acid side chains, as in voltage sensing (Armstrong, 1981), can be coordinated with the moving surfaces. Mechanisms that require extensive changes within a subunit or domain are less attractive, since the interiors of the folded polypeptide chains consist of tightly packed, interlocking side chains and do not provide enough flexibility to accommodate movements of more than 1 or 2 Å (Chothia and Lesk, 1985).

Bilayer-Spanning Structure

The portions of ion channels that are embedded in the lipid bilayer are composed of clusters of α-helices. At least this is what hydropathy analyses of the relevant polypeptides suggest. Usually there are four or more stretches of about 20 hydrophobic amino acids per subunit or domain: the correct length for an α-helix to span the hydrophobic lipid chains. Furthermore, the regular helical folding of the polypeptide chain facilitates hydrogen bonding between the polar backbone carbonyl (CO) and amide (NH) groups and so is a favored conformation in the hydrophobic surroundings. All things considered, the prediction of transmembrane α-helices based on hydropathy values is compelling.

It is nevertheless important to remember that the hydropathy scales are based largely on thermodynamic properties of the amino acids; they do not take into account the fact that polar residues may be required for stabilizing the structure or for functional purposes. Thus some transmembrane segments may be missed using

this approach (e.g., an amphipathic helix facing the pore). Furthermore, some pore-forming proteins in outer mitochondrial membranes and outer membranes of bacteria—VDAC channels and porins—have β-sheet instead of α-helix as the main transmembrane motif (Forte et al., 1987; Kleffel et al., 1985). It seems plausible that some of the "α-helical" channel proteins could include segments of β-sheet across the membrane as well.

How are α-helices packed together inside a bilayer? With soluble proteins, in which helices thread back and forth in roughly parallel and antiparallel directions, a commonly adopted motif is the four-α-helical bundle. Within this bundle, individual α-helices optimize interlocking of their amino acid side chains by twisting around each other in a left-handed sense, as in an α-helical coiled-coil (Crick, 1953; Cohen and Parry, 1986). Preferred interaxis angles made by one helix packed against the next are 10°-30°. Within the bilayer, the forces holding molecules together are different: polar interactions play a dominant role, and hydrophobic interactions no longer count. However, close packing must be optimized in the same way, and one would expect the same kind of twisting to occur. Indeed, this is observed. With bacteriorhodopsin (Henderson and Unwin, 1975), the helices twist around each other in a left-handed sense in six out of the seven pairwise contacts, making interaxis angles of up to 20°. With the L and M subunits of the photosynthetic reaction center (Deisenhofer et al., 1985) the sense of twisting is the same and the contacting helices are inclined to one another by even greater angles: 20°-30°.

Experimental data linking these bilayer-spanning structures to that of a channel protein have come from X-ray diffraction studies of isolated gap junctions. The X-ray pattern shows a strong reflection associated with a spacing in the protein of about 4.8 Å, shorter by 0.6 Å than the pitch of an α-helix (Makowski et al., 1982). A recent evaluation of the pattern suggests that this reflection arises from layers of side chains between bilayer-spanning helices, whose axes are inclined to one another (Tibbitts et al., 1989). With typical fibrous proteins, such as keratin and fibrinogen, whose structures are based on a coiled-coil, there is an equivalent meridional reflection at about 5.1 Å due to helices whose axes are inclined to each other by about 18°. The smaller value of 4.8 Å suggests that the channel helices may be even more strongly inclined, consistent with a proposal that they pack in the configuration of a four-α-helical bundle (Milks et al., 1988).

How do the twisted bundles associate together in a ring to create the ion channel? One consequence of this association is that the sense of twisting of the set of α-helices making subunit–subunit contacts (e.g., the pore-lining helices) must be opposite to that of helices coiled together within a subunit; that is, right-handed, instead of left-handed (Figure 4). Such intersubunit α-helical packing is found in the filamentous bacteriophage (Bryan et al., 1983) and in the multienzyme complex riboflavin synthase, in which case five helices from neighboring subunits associate together in a ring (Laden-
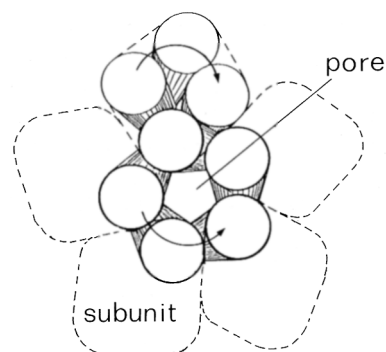

Figure 4. Twisting of Close-Packed α-Helices

The diagram shows the likely sense of twisting of helices within a subunit (left-handed, upper arrow) and of helices around a pore (right-handed, lower arrow). Only one of the five subunits (each composed of four α-helices) is shown in detail.

stein et al.,1988; Huber, 1989). In examples in which the right-handed twisting has been analyzed, the packing is usually accommodated by having a small residue from one of the pair of contacting helices facing a large residue from the other (Chothia et al., 1981). The putative pore-lining helices contain parallel ridges of small and large residues, which could be involved in interactions of this sort (see below).

The Ion Pathway

Channels are compromising molecules. A high permeability is favored by a wide pathway for the ions, whereas a high selectivity requires it to be narrow. What is the design that most satisfactorily reconciles these conflicting properties within a stable, bilayer-spanning structure? The profile of the acetylcholine receptor channel at a resolution of 17 Å (Figure 5) provides some clues. There are two 20–25 Å wide openings to this channel—one extending ~60 Å from the outer membrane surface into the synaptic cleft and the other ~20 Å from the inner membrane surface toward the cell interior. Between these openings, and passing through the hydrophobic core of the bilayer is a much narrower portion (about 30 Å long and no more than 10 Å wide), which would present the greatest physical barrier to the ions. The partitioning is quite abrupt at the level of the phospholipid headgroups (probably reflecting changes in the internal folding of the protein) and so the ion pathway is divided distinctly into a narrow pore bracketed by two wide entrance domains. Why have so much of the channel protein—almost three-quarters of the total length—protruding so far outside the membrane?

The extra length is more than is needed for the receptor to protrude clear of other molecules on the postsynaptic membrane surface (Heuser and Salpeter, 1979; Bridgman et al., 1987) and thus be freely accessible to cations and ligands. Also, this design cannot be something unique to the acetylcholine receptor: other channels of the same superfamily are composed of homologous polypeptide chains of similar length and therefore
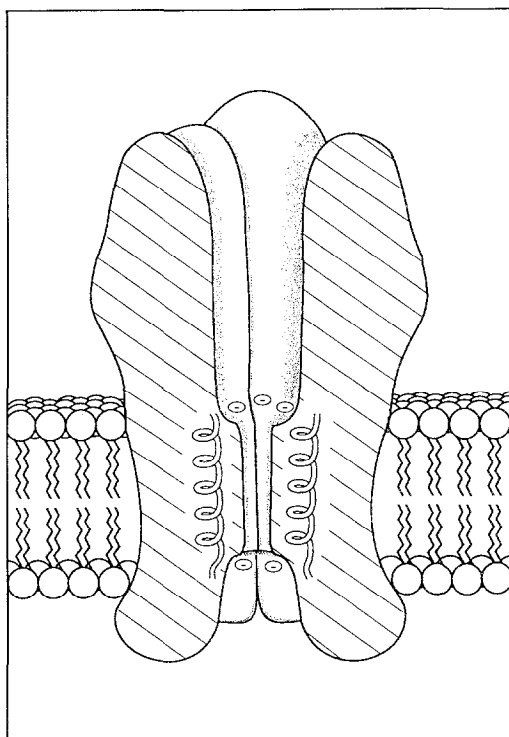
Figure 5. Profile of a Cation-Selective Channel

The schematic picture is based on the three-dimensional structure of the acetylcholine receptor determined by helical reconstruction from cryo-images of Torpedo postsynaptic membranes (Toyoshima and Unwin, 1988). The walls lining the entrances probably contain an excess of negatively charged groups (positively charged groups in the case of anion-selective channels). A part of the α-helical segment M2 from each subunit lines the narrow pore spanning the hydrophobic portion of the bilayer. The ring of pore-lining α-helices probably forms a coiled structure as depicted in Figure 4 (see text). Rings of negatively charged groups near or at the ends of M2 (Imoto et al., 1988) are probably close to or at the regions where the pore widens abruptly into the entrances. The cytoplasmic end of the structure is at the bottom.

Table 1. Net Charges on Extracellular Portions of Cation- and Anion-Selective Channels

	Extracellular	Cytoplasmic	Total
Cation-Selective			
ACh R α[a]	8(−)	4(−)	12(−)
AChR β[b]	13(−)	4(−)	17(−)
AChR γ[c]	17(−)	2(+)	15(−)
AChR δ[b]	11(−)	1(+)	10(−)
Neuronal AChR α3[d]	10(−)	3(+)	7(−)
Neuronal AChR β2[e]	10(−)	3(+)	7(−)
Anion-Selective			
GABA α[f]	2(+)	7(+)	9(+)
GABA β[f]	2(−)	7(+)	5(+)
GABA γ2[g]	1(+)	5(+)	6(+)
Glycine receptor[h]	1(−)	9(+)	8(+)

Calculations were made assuming four transmembrane segments (Claudio et al., 1983; Devillers-Thiery et al., 1983; Noda et al., 1983).
[a] Noda et al., 1982.
[b] Noda et al., 1983a.
[c] Noda et al., 1983b.
[d] Boulter et al., 1986.
[e] Deneris et al., 1988.
[f] Schofield et al., 1987.
[g] Pritchett et al., 1989.
[h] Grenningloh et al., 1987.

have essentially the same quaternary structure. However, all channels in the superfamily do have in common a particularly high permeability (typically transporting more than ∼10^4 ions per ms) as well as a high selectivity for either cations (acetylcholine receptors) or anions (GABA$_A$ and glycine receptors). So a likely possibility is that the extended entrances are there in order to combine these two properties in the most effective way.

Some evidence that the entrances have a role in enhancing selectivity comes from comparisons of net charges contributed by amino acid residues outside the bilayer-spanning portions of different cation- and anion-selective channels (Schofield et al., 1987). Both the extracellular and cytoplasmic portions of the cation-selective channels contain a net negative charge or a slight net positive charge, whereas the equivalent portions for the anion-selective channels are consistently more positive (Table 1). Although in the absence of a high resolution structure, the charged groups lining the wall of the channel cannot be distinguished from those in other

places, the overall differences between the two types of channel, summed over five subunits, are striking. They seem to indicate that the walls lining the entrances of the cation- and anion-selective channels may contain significant excesses of negatively and positively charged residues, respectively.

Clusters of like charges have a strong local effect on the relative concentrations of anions and cations, and the implication is that the charged amino acid residues are used by channels to concentrate the ions they select for and dilute out the ions they discriminate against. The ionic environment would then be modified close to the narrow part of the channel, particularly if the charged residues are concentrated in that vicinity (Dani and Eisenman, 1987; Jordan, 1987), making it easier for the selected ions (together with bound water molecules) to diffuse through. These ions would probably be stabilized transiently by polar residues and backbone groups lining the narrow part, and pairing interactions with diffusing counter-ions would compete with this process.

The above arguments apply especially to the synaptic entrances of channels in the acetylcholine receptor superfamily, in which the flux of ions (cations or anions) is mostly toward the cell interior from the outside. But why have a long cylindrical entrance, rather than a shorter funnel-shaped opening, as is often depicted in sketches? Probably the main reason is that funnels, which widen rapidly from a narrow pore, spread the electric field, and tend to place the charged residues too far away to have much influence. Cylindrical entrances, on the other hand, would tend to concentrate the electric field and keep charges within the Debye distance (about 10 Å at physiological ion concentrations) so that

they can exert a strong local effect. The cylindrical opening of the acetylcholine receptor does not limit the flux of ions across the membrane because the radius (which is more important than length) is large (10–12 Å) throughout. In essence, it seems that the cylindrical entrance is designed to be sufficiently wide to present no appreciable barrier for diffusion and sufficiently narrow that residues lining the wall can exert a significant electrostatic effect.

Do ion selective channels in other superfamilies have a similar entrance design? This seems likely according to the arguments just given, and may partly account for the fact that most channel proteins appear to have more than three-quarters of their total mass excluded from the transmembrane segments, implying that they protrude substantially into the water.

Pore-Lining Segments

It is often assumed that α-helical segments from neighboring subunits associate to line the bilayer-spanning portion of a channel (Guy, 1984; Finer-Moore and Stroud, 1984; Greenblatt et al., 1985; Guy and Seetharamulu, 1986). This makes sense if one takes the observed profile (Figure 5), in which the bilayer-spanning portion is the narrowest, and then attempts to correlate pore size with the number of encircling α-helices. Highly selective channels, such as the voltage-gated sodium and calcium channels, have the smallest pores and would also have the smallest number of encircling helices (four); less selective channels with intermediate-sized pores, such as the acetylcholine receptor, would have one more helix (five); and very weakly selective channels, such as the gap junction channel, would have the most (six).

Structures determined from soluble proteins, such as the four-α-helical bundle of myohemerythrin (Hendrickson et al., 1975), where there is no room for a pore, and the five-α-helical ring in riboflavin synthase, where the opening is about 9 Å across (Ladenstein et al., 1988), would argue against a more quantitative consideration. They stress that parameters such as tilting of the helices, the size of residues involved in their side-to-side packing, and the nature of the projecting side chains could introduce a broad range of possibilities. On the other hand, inspection of relevant amino acid sequences (see below) suggests that the diversity among channels in these parameters may be rather small.

Several lines of evidence have suggested that at least part of the putative transmembrane helix M2 of the acetylcholine receptor lines the open pore. First, experiments conducted on chimeric calf/Torpedo δ subunits demonstrated that a stretch of the sequence composed of M2 and flanking residues is involved in determining the rate of ion transport (Imoto et al., 1986). Second, noncompetitive blockers of the open channel, chlorpromazine and triphenylmethylphosphonium, were shown to photolabel a serine residue on M2, in homologous positions on the α, β, and δ subunits, while retaining a 1:1 stoichiometry with the whole complex (Giraudat et al., 1986, 1987; Hucho et al., 1986). Third, directed muta-

genesis of M2s of mouse acetylcholine receptors at the site corresponding to this serine residue modified both the single-channel conductance and the equilibrium binding affinity of an open channel blocker, QX-222 (Leonard et al., 1988). Fourth, experiments in which negatively charged glutamic or aspartic acid residues located at or near the ends of M2 (see Figure 5) were mutated to neutral or positive residues demonstrated that charges at these sites had (in the absence of divalent cations) a marked effect on the rate of ion transport (Imoto et al., 1988). Most significant was the ring of charges only 7 residues away (toward the cytoplasm) from the labeled serines.

The arguments applying to the acetylcholine receptor must apply to other members of the same superfamily and possibly to the ryanodine receptor, which has a similar amino acid sequence along its assigned M2 (Takeshima et al., 1989). With other superfamilies, there is no direct evidence to indicate which bilayer-spanning segment is the most likely candidate to line the pore. However, in the case of the gap junction channel the segment M3 is the only reasonable candidate, since it is the only segment having a pronounced amphipathic character (Milks et al., 1988). The same can be said for M6 of MIP, a channel-forming protein in the lens (Gorin et al., 1984; Zampighi et al., 1985). In the case of the voltage-gated sodium channel (and other members of this superfamily), several possible segments have been suggested. One is S3, since the peptide corresponding to this segment, when incorporated into lipid bilayers, mimics some of the gating characteristics of the authentic channel (Oiki et al., 1988a). However, the peptide corresponding to S4 (the "voltage sensor"; Stuhmer et al., 1989) also forms channels in bilayers (Tosteson et al., 1989), and an unequivocal assignment cannot be made.

Figure 6 compares amino acid sequences of some putative pore-lining helices, including those belonging to different channel classes. All of the sequences contain a similar pattern of residues in the half closest to the amino terminus, that is the half (with the exception of MIP and the ryanodine receptor; Gorin et al., 1984; Takeshima et al., 1989) thought to be closest to the cytoplasm. In particular, small polar or neutral residues are repeated in every fourth position, occasionally several times. These residues, which include the labeled serines of the acetylcholine receptor, make a continuous ridge on the helix surface (Figure 7). The ridge slopes up to the right at an angle of almost 30° to the helix axis. However, to optimize its packing, the helix itself is probably twisted, in a left-handed sense, around other helices of the same subunit (Figure 4). This would make the ridge align more nearly parallel to the axis of the channel. In other words, it is probably the whole ridge, not just the labeled serines (or equivalent residues), that is exposed to water inside the open pore—a deduction consistent with recent experiments demonstrating modulation of QX-222 binding by a second residue on the ridge (see Figure 7; Charnet et al., 1990).

The ridge of small polar residues is also flanked on the right by a ridge of large hydrophobic residues (Figures 6

channel	segment	subunits	amino acid sequence
connexin32 (1)	M3	6	T L W W T Y V I S V V F R L L F E A V F M Y V
connexin43 (2)	M3	6	G L L R T Y I I S I L F K S V F E V A F L L I
synaptophysin (3)	M3	6	G P M M D F L A T A V F A F M W L V S S S A W
AchR, alpha (4)	M2	5	G E K M T L S I S V L L S L T V F L L V I V E
AchR, beta (5)	M2	5	G E K M S L S I S A L L A V T V F L L L L A D
AchR, gamma (6)	M2	5	G Q K C T L S I S V L L A Q T I F L F L I A Q
AchR, delta (5)	M2	5	G E K M S T A I S V L L A Q A V F L L L T S Q
neur. AchR, alpha3 (7)	M2	5	G E K V T L C I S V L L S L T V F L L V I T E
neur. AchR, beta2 (8)	M2	5	G E K M T L C I S V L L A L T V F L L L I S K
GABA rec., alpha (9)	M2	5	P A R T V F G V T T V L T M T T L S I S A R N
GABA rec., beta (9)	M2	5	A A R V A L G I T T V L T M T T I S T H L R E
GABA rec., gamma2 (10)	M2	5	P A R T S L G I T T V L T M T T L S T I A R K
glycine rec. (11)	M2	5	P A R V G L G I T T V L T M T T Q S S G S R A
MIP (12)	M6	?	V Y W V G P V I G A G L G S L L Y D F L L F P
ryanodine rec. (13)	M2	4	M E P A L W C L S L L H T L V A F L C I I G Y

Figure 6. Amino Acid Sequences of Putative Pore-Lining α-Helices

Shown are the sequences from a number of channels in the different classes (Figure 1), their identifying transmembrane segments, and the number of their subunits (inferred in some cases, rather than determined directly). There are similar patterns of residues, mainly in the half (left) closest to the amino terminus. Boxes are drawn around small polar or neutral residues that repeat at every fourth position and around some neighboring large hydrophobic residues (broken lines) that could be important in gating (see text). The flexible lysine and arginine side chains are included in the boxes because they can create spaces in the helix ridges and, in effect, substitute for a smaller residue; proline residues are also included because they may play a similar role. The standard one-letter amino acid code is used. Numbers in parentheses denote references: (1) Kumar and Gilula, 1986; Paul, 1986; (2) Beyer et al., 1987; (3) Buckley et al., 1987; Leube et al., 1987; Thomas et al., 1988; (4) Noda et al., 1982; (5) Noda et al., 1983a; (6) Noda et al., 1983b; (7) Boulter et al., 1986; (8) Deneris et al., 1988; (9) Schofield et al., 1987; (10) Pritchett et al., 1989; (11) Grenningloh et al., 1987; (12) Gorin et al., 1984; (13) Takeshima et al., 1989.
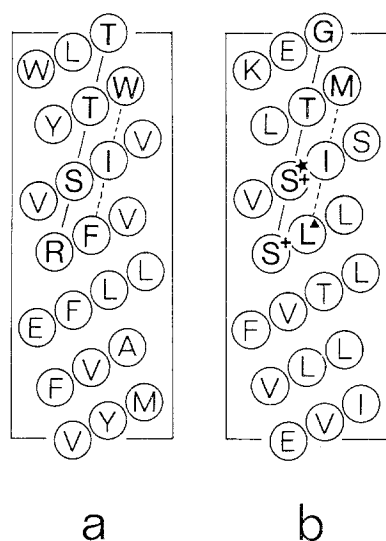
Figure 7. Examples of Putative Pore-Lining α-Helices

(a) M3 of connexin32, forming a hexameric channel. (b) M2 of the α subunit of the Torpedo acetylcholine receptor, forming a pentameric channel. There is a common alignment of small residues (connected by full lines), flanked on the right by a line of large hydrophobic residues (connected by broken lines); the hydrophobic residues, isoleucine and leucine, are strongly conserved in the five-subunit class; the leucines are replaced by phenylalanines in the six-subunit class. The star in (b) denotes the serine residue labeled by chlorpromazine and triphenylmethylphosphonium (Giraudat et al., 1986, 1987; Hucho et al., 1986); the plus sign denotes the two positions where mutations affect the binding of QX-222 (Leonard et al., 1989; Charnet et al., 1990); the triangle denotes the leucine residue that, in the β subunit of the acetylcholine receptor, is labeled by chlopromazine (Giraudat et al., 1987). The cytoplasmic ends of the helices (toward the amino terminus) are uppermost.

and 7) of which isoleucine and leucine (in the five-subunit class) and phenylalanine (in the six-subunit class) are highly conserved. The leucine is the only invariant residue among forty different sequences of M2 from various polypeptides in the acetylcholine receptor superfamily and so appears to be particularly important. Chlorpromazine labeling of this residue on the β subunit of the acetylcholine receptor (Giraudat et al., 1987) suggests that it at least partly lines the pore.

These aligned residues identify a special region of the pore, 10–15 Å in extent, common to all of the channel classes considered. At the corresponding level of the structure, the pore-lining helices must contact one another, probably narrowing the pore to its minimal dimensions. The absence of a conserved repeating pattern of residues in other regions and packing considerations (Furois-Corbin and Pullman, 1989) suggest that the helices elsewhere may be spaced farther apart.

Gating Mechanism

Very little information has been forthcoming about how ion channels open and close. Biophysical and electrophysiological experiments have told us mainly about the events associated with gating, such as the cooperativity of ligand binding and transitions involving charge movements across the membrane, but not so much about the physical nature of the action itself. Directed mutagenesis, combined with functional study, addresses more readily other questions such as the factors governing rate of ion transport, duration of single-channel open times, and ion selectivity. The paucity of direct structural
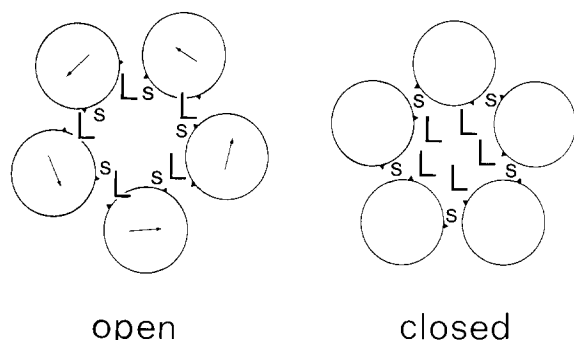
open   closed

Figure 8. Hypothetical Model for Closing a Pore Formed by α-Helical Segments from Neighboring Subunits

The open pore is lined mainly by small residues (S) and the backbone CO and NH groups contributed by the encircling helical segments. Closure is achieved by small, concerted displacements (arrows), produced by tilting of the subunits, which move in large hydrophobic residues (L) to fill up the space. With five encircling subunits, isoleucine and leucine (which extend radially almost 8 Å from the helix axis) should be sufficient to fill the space. With six encircling subunits, bulkier aromatic residues such as phenylalanine (which extends ∿9 Å from the helix axis) would probably be required. With homologous subunits, rather than identical ones (as drawn), a similar but less symmetrical rearrangement might be involved.

data becomes most evident when it comes to discussing mechanisms.

Nonetheless, two structural findings of a different kind point to a similar location for the gate. First, the narrow portion of the acetylcholine receptor channel is confined just to the region of the bilayer, suggesting that the gate of the receptor is at a level inside the phospholipid headgroups. Second, the structural changes affecting the pore of the gap junction channel are greatest close to the cytoplasmic membrane surface, favoring a location for the gate near there. With either channel, the gate could well be at the level of the cytoplasmic leaflet of the bilayer, implicating the region of the pore-lining helices where the small polar and large hydrophobic residues are aligned.

One reason to suppose that residues on the helical segments could participate in gating is the observation that, when inserted into lipid bylayers by themselves, segments, such as M2 of the acetylcholine receptor, are able to mimic both the conductance and the gating characteristics of the authentic channels (Oiki et al., 1988a, 1988b). This is also true of synthetic peptides containing different combinations of small polar and large hydrophobic residues (Lear et al., 1988). It is assumed that these peptides form symmetrical oligomers of α-helices in the bilayer, with the polar residues pointing inward to create the hydrophilic pathway for the ions. But how do these artificial pores close? Are there transient cooperative rotations of individual helices, which bring the hydrophobic residues inward to block them? Or is there instead some rapid assembly/disassembly process? Answers to these questions may shed some light on the gating process of the authentic channels. On the other hand, there could be some basic

differences between the pores created by peptides and those created by the authentic channels. For example, the peptide helices are free to adopt the favored packing of a left-handed supercoil, whereas those integrated into the folded polypeptides are not.

What is the physical nature of the gate? The ease with which functional channels can be made from the peptides, and more significantly, from hybrid and mutated receptors, suggests that the ability of a pore to close completely is not determined by a single residue or a ring of residues. Rings of charged residues that affect the rate of ion transport and could be involved in gating are not conserved among different members of the same superfamily. So these data argue against a gating mechanism that would involve specific interactions between a set of residues to create a bridge across the pore. The specificity and accuracy characteristic of enzyme–substrate interaction are not needed to shut off the flow of ions.

An alternative possibility is that channels create a physical block by a structural change in which larger residues are moved to replace smaller ones lining the wall of the pore. Such a steric mechanism (in contrast to an electrostatic mechanism) would account for the fact that channels, such as the acetylcholine receptor, seem to form a seal tight enough to prevent the passage of small, uncharged molecules as well as ions when they are closed (Huang et al., 1978).

The strikingly conserved alignment of small polar and large hydrophobic residues along the pore-lining α-helices suggests how this block might occur in essentially the same way for all classes of channel (Figure 8). One assumes that the ridge of small residues (S), together with the backbone CO and NH groups, lines the wall of the open pore. The open pore is then filled by moving concertedly into their place the large hydrophobic residues (L), while burying the small residues at the side. Tilting rearrangements of the sort described earlier could produce these movements, and it is notable that the observed sense of rotation to narrow a pore (Figure 2, arrows) is the correct one to bring the large residues into the center. The resulting closed configuration might be quite stable because of hydrogen bonding of the polar serine and threonine residues to the backbone CO groups (Gray and Matthews, 1984) or to other buried residues, rather than to water, and because of hydrophobic interactions between the large residues, which are now juxtaposed.

Conclusions

In recent years many channels have been discovered, characterized by single-channel conductance measurements, and described completely in terms of their primary sequence. Some superfamilies of channels have emerged, and undoubtedly others will follow. There has been a glimpse of how channels are put together in three-dimensions, and some clues about conformational changes in switching between different functional states have been obtained.

An overview of some of the best-characterized chan-

nels suggests that there may be unifying themes among them, extending beyond the fact that they have the same symmetrical architectural plan. Channels of different superfamilies, with different numbers of subunits, share identical alignments of small polar and large hydrophobic residues along their putative pore-lining α-helices, suggesting that they may incorporate similar packing arrangements and work by similar mechanisms. It may be a general rule that the narrower portions of channels are formed by close associations of α-helices contributed by each of the encircling subunits and that the number depends mainly on the size of pore needed to suit the task.

The structural details of an ion pathway seen in just one example are hardly enough to go by. But it would not be surprising if the narrow portions of all ion-selective channels are bracketed at the level of the phospholipid headgroups by wide entrances protruding well into the water, so that additional surfaces can be used to enhance selectivity and efficiency of ion transport. Specificity in protein–protein or protein–nucleic acid interactions is often accomplished by weak influences in different regions of a structure adding up to produce a strong effect overall, and it could be that ion-selective channels, to a greater or lesser extent, utilize this same principle.

There is some evidence that switching of conformations to open and close channels, or to inactivate them, involves small tilting rearrangements of the subunits. The ease with which functional hybrid molecules can be assembled suggests that the subunit–subunit contacts do not involve extensive interactions as would prevent such action. Tilting rearrangments about axes parallel to the membrane plane would be particularly favored because they mediate changes of structure that can be readily accommodated in the two phase water/lipid environment.

Acknowledgments

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