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Molecular Properties of Sodium and Calcium Channels

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Voltage-gated sodium and calcium channels are responsible for inward movement of sodium and calcium during electrical signals in cell membranes. Their principal subunits are members of a gene family and can function as voltage-gated ion channels by themselves. They are expressed in association with one or more auxiliary subunits which increase functional expression and modify the functional properties of the principal subunits. Structural elements which are required for voltage-dependent activation, selective ion conductance, and inactivation have been identified, and their mechanisms of action are being explored through mutagenesis, expression in heterologous cells, and functional analysis. These experiments reveal that these two channels are built on a common structural theme with variations appropriate for functional specialization of each channel type.

KEY WORDS: Ion transport; action potential; ion channels; calcium; sodium; voltage-dependent gating.

The sodium and calcium channels are perhaps the most similar of the major families of ion channels in their overall structure. The amino acid sequences of their principal subunits are 25 to 30% identical, but the sequence identity is much higher in the transmembrane segments which contain many of the core functional elements of the channels. In contrast, the amino acid sequences are very divergent in the hydrophilic domains, and the principal subunits of sodium and calcium channels associate with distinct sets of auxiliary subunits which also influence their function in critical ways. During the evolution of sodium and calcium channels, structural specializations have been introduced to serve specific functional requirements in cellular physiology. This article focuses on the structure and function of sodium and calcium channels and compares the structural elements which are responsible for specific aspects of channel function.

SUBUNIT STRUCTURE OF SODIUM AND CALCIUM CHANNELS

Sodium channels have at least five separate receptor sites for neurotoxins which have different effects on channel function (Table I) (Catterall, 1988a; Strichartz *et al.*, 1987). Sodium channels purified from rat brain using the high-affinity binding of the pore blocker saxitoxin as a specific label consist of three glycoprotein subunits [α (260 kDa), β1 (36 kDa), and β2 (33 kDa)] in 1:1:1 stoichiometry in a complex of 320 kDa as illustrated in Fig. 1 (Catterall, 1988b). The β2 subunit is linked to α by disulfide bonds while β1 is noncovalently attached.

Table I. Neurotoxin Receptor Sites on the Sodium Channel

Site	Toxin	Effect
1	Tetrodotoxin Saxitoxin μ-Conotoxins	Inhibition of ion conductance
2	Veratridine Batrachotoxin Aconitine Grayanotoxin	Persistent activation
3	α-Scorpion toxins Sea anemone toxins	Inhibit inactivation; enhance persistent activation
4	β-Scorpion toxins	Shift voltage dependence of activation
5	Brevetoxins Ciguatoxins	Repetitive firing; shift voltage dependence of activation

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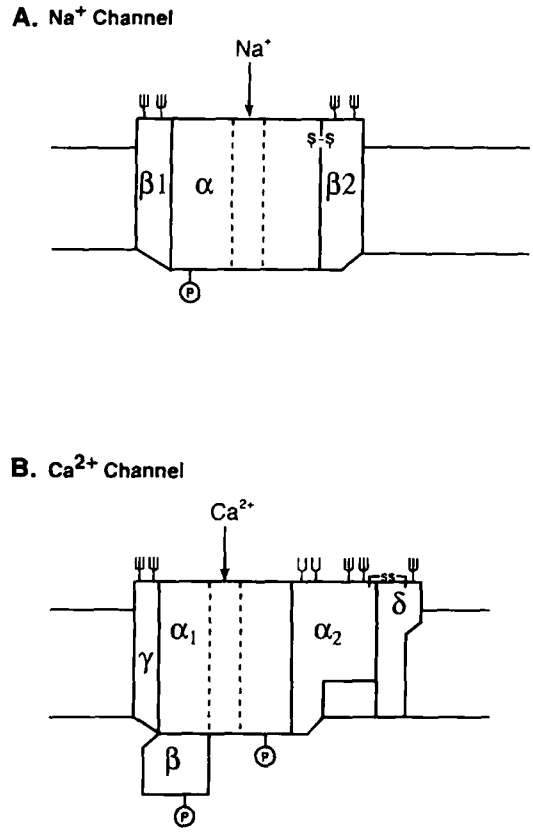

Fig. 1. Subunit structures of the voltage-gated ion channels. The arrangement and biochemical properties of subunits of the voltage-gated ion channels are illustrated. Ψ, site of probable N-linked glycosylation; P, site of cAMP-dependent protein phosphorylation; -S-S-, inter-subunit disulfide bond.

The α subunit is a transmembrane glycoprotein which binds neurotoxins on its extracellular surface and can be phosphorylated by protein kinases on its intracellular surface. The β1 and β2 subunits are integral membrane glycoproteins. Sodium channels in skeletal and cardiac muscle have α and β1 subunits but not β2 subunits (Gordon *et al.*, 1988; Barchi, 1988), while sodium channels from eel electroplax have only α subunits (Agnew, 1984).

The complete primary structures of the α subunits of sodium channels from electric eel electroplax and rat brain, skeletal muscle, and heart have been deduced from cDNA sequence (Noda *et al.*, 1984, 1986a; Kayano *et al.*, 1988; Trimmer *et al.*, 1989; Auld *et al.*, 1988; Kallen *et al.*, 1990; Rogart *et al.*, 1989). Each consists of four internally homologous domains with six proposed alpha helical transmembrane segments, S1-S6, based on computer analysis of hydrophobicity (Fig. 2). α Subunit mRNA, purified by hybrid selection or transcribed from cDNA, is sufficient to direct the synthesis of functional sodium channels in *Xenopus* oocytes, although their inactivation rate and gating properties are altered (Trimmer *et al.*, 1989; Auld *et al.*, 1988; Goldin *et al.*, 1986; Noda *et al.*, 1986b). Transfection of cDNA encoding the α subunit in mammalian cells results in expression of sodium channels with normal physiological properties and normal response to neurotoxins acting at receptor sites 1 through 3 (Scheuer *et al.*, 1990). Evidently, the principal α subunits of sodium channels are functionally autonomous in mediating voltage-dependent ion conductance that is modulated by drugs and neurotoxins.

Voltage-gated calcium channels are present in high concentration in the transverse tubules of vertebrate skeletal muscle. The principal voltage-gated calcium channels in muscle are L-type; they mediate long-lasting calcium currents in response to strong depolarizations. They are inhibited by a variety of pharmacological agents used in the treatment of cardiovascular disorders including the dihydropyridines and the phenylalkylamines. The transverse tubule L-type calcium channels have been purified using their high-affinity binding of dihydropyridine calcium channel antagonists as a specific label. These channels are a complex of five protein subunits (Fig. 1) (Catterall *et al.*, 1988; Campbell *et al.*, 1988; Glossmann and Striessnig, 1990). The α1 subunit is the central functional component of the complex. Its mRNA encodes a protein of 212 kDa having four homologous domains with six proposed alpha helical transmembrane segments in each like sodium channel α subunits (Fig. 2) (Tanabe *et al.*, 1987). Distinct genes encode α1 subunits that are expressed in heart, smooth muscle, and brain (Mikami *et al.*, 1989; Biel *et al.*, 1990; Snutch *et al.*, 1990). The α1 subunit contains the receptor sites for calcium channel antagonists (Catterall *et al.*, 1988; Campbell *et al.*, 1988; Glossmann and Striessnig, 1990) and can function as a voltage-gated ion channel when expressed in appropriate recipient cells (Mikami *et al.*, 1989; Perez-Reyes *et al.*, 1989). Two size forms of the α1 subunit having apparent molecular masses of 212 and 175 kDa have been identified (De Jongh *et al.*, 1989). The 175-kDa form accounts for more than 90% of α1 subunits in purified preparations and transverse tubule membranes; both forms are present in skeletal muscle cells in cell culture (Lai *et al.*, 1990). α1 Subunits are present in a complex with

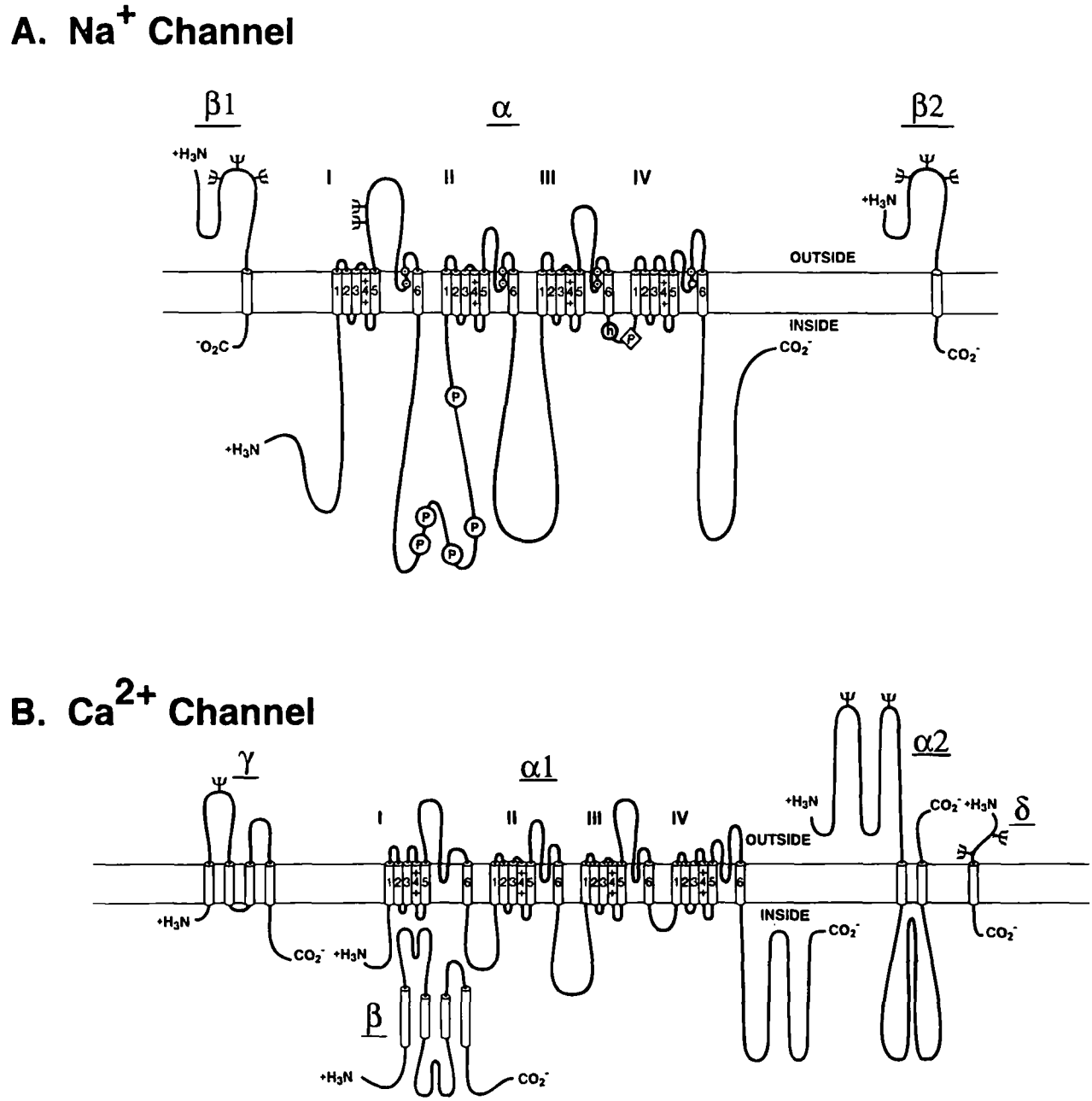

Fig. 2. Transmembrane organization of the ion channel subunits. The primary structures of the subunits of the voltage-gated ion channels are illustrated. Cylinders represent probable alpha helical segments. Bold lines represent the polypeptide chains of each subunit with length approximately proportional to the number of amino acid residues. Ψ, sites of probable N-linked glycosylation; P, sites of demonstrated protein phosphorylation.

an intracellularly disposed β subunit of 55 kDa and a glycosylated transmembrane γ subunit of 30 kDa (Catterall *et al.*, 1988; Campbell *et al.*, 1988; Glossmann and Striessnig, 1990) which are encoded by separate genes (Ruth *et al.*, 1989; Jay *et al.*, 1990). The α1, β, and γ subunits also interact with a disulfide-linked glycoprotein complex of α2 and δ subunits. These two subunits are encoded by the same gene (Ellis *et al.*, 1988) whose protein product is proteolytically processed at position 934 to yield the disulfide-linked α2 and δ polypeptides (De Jongh *et al.*, 1990).

STRUCTURE AND FUNCTION OF THE PRINCIPAL SUBUNITS OF SODIUM AND CALCIUM CHANNELS

Purification, molecular cloning, and determination of the primary structures of the principal subunits of sodium, calcium, and potassium channels has provided a molecular template for probing the relationship between their structure and function (reviewed in). The structure of the principal subunits of each of these channels is based on the same motif: four homologous transmembrane domains which contain six probable transmembrane alpha helices and surround a central ion pore. Although each channel contains associated auxiliary subunits, the principal subunits can carry out the basic functions of the voltage-gated ion channels by themselves and therefore must contain the necessary structural elements for ion channel function within them.

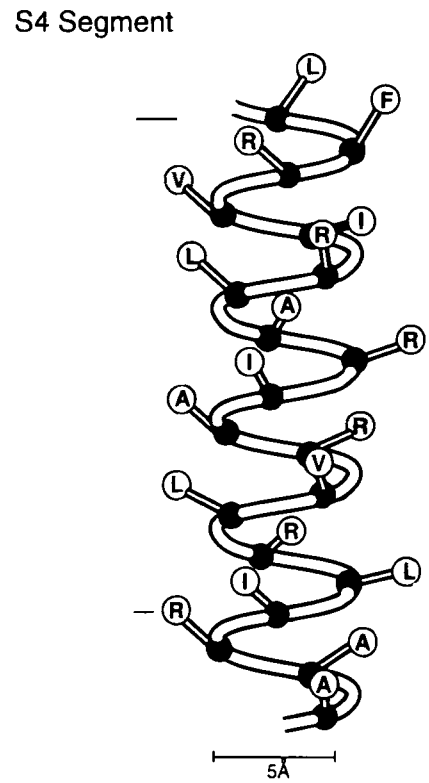

Fig. 3. The S4 segment from domain IV of the sodium channel is illustrated in a ball-and-stick alpha helical representation. Amino acids are indicated in single letter code and the positively charged amino acids in every third position are numbered from the extracellular end of the helix.

VOLTAGE-DEPENDENT ACTIVATION

Activation of the voltage-gated ion channels is thought to result from a voltage-driven conformational change which opens a transmembrane pore through the protein. Depolarization of the membrane exerts an electrical force on voltage sensors which contain the gating charges of the channel located within the transmembrane electrical field. These gating charges are likely to be charged amino acid residues located in transmembrane or membrane-associated segments of the protein. The movement of the gating charges of the sodium channel through the membrane driven by depolarization has been directly measured as an outward gating current (Armstrong, 1981). The gating current for sodium channels was initially estimated to correspond to the movement of approximately six charges all the way across the membrane; movement of a larger number of charges across a fraction of the membrane electric field would be equivalent. More detailed analyses of potassium channel gating currents suggest substantially larger gating charge movements in the range of 12 charges per channel. Identification of the voltage sensors and gating charges of the voltage-gated ion channels is the first critical step toward understanding the molecular basis of voltage-dependent activation.

Inspection and analysis of the primary structure of the sodium channel α subunit, the first member of the voltage-gated ion channel gene family, led to the prediction that the fourth transmembrane segment in each domain (the S4 segment) might serve as the voltage sensor (Catterall, 1988b; Numa, 1989; Guy and Conti, 1990) (Fig. 3B). The prediction that these positive charges serve as gating charges has been tested by mutagenesis and expression studies of both sodium and potassium channels. In each case, the gating charge has been inferred from measurements of the steepness of voltage dependence of channel activation at low levels of activation where this provides an indirect and approximate estimate of gating charge.

Neutralization of the four positively charged residues in the S4 segment of domain I of the sodium channel by site-directed mutagenesis has major effects on the voltage dependence of activation (Stühmer *et al.*, 1989). Neutralization of the arginine residue in position 1 (R1) had little effect on the steepness of sodium channel activation, but neutralization of the positively charged residues in positions 2 through 4 in this S4 segment reduced the apparent gating charge by 0.9 to 1.8 charges. Combined neutralization of mul-

tiple charged residues and mutations of positive charges to negative charges causes progressively increasing reduction of gating charge, but the reduction of apparent gating charge is less than proportional to the expected reduction in total charge of the S4 segment. In addition, most of the mutations also caused shifts of the voltage dependence of activation to more positive or more negative membrane potentials.

If the S4 segments must move through the protein structure during the process of activation, the size and shape of the hydrophobic residues in these segments should also have an important influence on voltage-dependent activation. Mutation of a single leucine residue to phenylalanine in an S4 segment of a sodium channel shifts the voltage dependence of gating 20 mV (Auld *et al.*, 1990). These results, together with the effects of charge neutralization mutations, provide strong support for identification of the S4 segments as the voltage sensors of the voltage-gated ion channels and for identification of the positively charged residues within them as the gating charges of the channel.

S3 segments and the adjacent linker region connecting to S4 have also been implicated as key determinants of the activation gating of calcium channels. The calcium channels expressed in skeletal muscle, which contain α_{1S}, activate much more slowly than the closely related calcium channels in heart, which contain α_{1C}. Analysis of chimeric α_1 subunits revealed that this gating behavior was determined by the S3 segments in domain I and the adjacent extracellular loop connecting S3 and S4 (Nakai *et al.*, 1994). Neither segment alone was sufficient to transfer the difference in gating kinetics between these two channels. Thus, the S4 segments and adjacent sequences are implicated in determination of the gating characteristics of different ion channels as well as in the basic mechanism of activation gating itself. The S3 segments may interact with the gating charges of the S4 segments during the gating process.

The mechanism by which the S4 segments serve as voltage sensors is not known. The "sliding helix" or "helical screw" models (Catterall, 1988b; Guy and Conti, 1990) proposed that the entire S4 helix moves across the membrane along a spiral path exchanging ion pair partners between its positively charged residues and fixed negative charges in surrounding transmembrane segments. This model implies a large (but unknown) energy barrier for breaking and re-making numerous ion pairs within the protein structure and suggests an approximate equivalence of gating charge movement among the different charged residues in the S4 helices. Because neutralization of individual charged residues has very different effects on the voltage dependence of channel activation, it is unlikely that this simple model can be correct in detail. A more complex "propagating helix" model proposes that the S4 transmembrane segments undergo an alpha helix–beta sheet transition which propagates outward to move charged residues across the membrane (Guy and Conti, 1990). This model also has no direct experimental support, but it has the potential to accommodate at least some of the differences observed among individual residues because not all charged residues in the S4 segment are proposed to move the same distance across the membrane. An important result in favor of models in which the S4 segments move outward across the membrane during the gating process was recently derived from studies of a naturally occurring mutation in the human skeletal muscle sodium channel. Mutation of an arginine residue at the extracellular end of the S4 segment in domain IV to cysteine impairs channel inactivation, suggesting that this segment is involved in coupling of activation to inactivation (Chahine *et al.*, 1994). During the activation process, the cysteine residue in this mutant becomes available to hydrophilic cysteine reagents applied from the extracellular side of the membrane (Yang and Horn, 1995). These results support the hypothesis that the S4 transmembrane segment moves outward during the gating process. Thus, the S4 segments are clearly implicated as the voltage sensors of the voltage-gated ion channels and may move during the conformational change to the activated state, but the mechanism through which they initiate activation of the channels remains unknown.

THE TRANSMEMBRANE PORE AND THE RECEPTOR SITES FOR PORE BLOCKERS

Essentially all models for the structure of the voltage-gated ion channels include a transmembrane pore in the center of a square array of homologous transmembrane domains. Each domain would contribute one-fourth of the wall of the pore. Identification of the segments which line the transmembrane pore and define the conductance and ion selectivity of the channels is of great interest and importance. A number of toxins, drugs, and inorganic cations are blockers of the voltage-gated ion channels. In several cases, detailed biophysical analysis of their mechanism of action indicates that these molecules enter and bind within the transmembrane pores of the channels and

compete with permeant ions for occupancy of the pore (Hille, 1992). These channel blockers therefore serve as molecular markers and specific probes of the pore region of the ion channels. Amino acid residues which form the extracellular and intracellular mouths of the transmembrane pores have been identified by their interaction with pore-blocking drugs and toxins.

The Extracellular Mouth of the Pore

Tetrodotoxin and saxitoxin are thought to block sodium channels by binding with high affinity to the extracellular mouth of the pore (Hille, 1992). Their binding is so specific that they were used as a marker in the initial purification of sodium channels from excitable cell membranes. Block of their binding by protonation or covalent modification of carboxyl residues led to the model that these cationic toxins bind to a ring of carboxyl residues at the extracellular mouth of the pore (Hille, 1992). These residues have now been identified by site-directed mutagenesis. Noda *et al.* (1990) neutralized glu387 in rat brain sodium channel II by mutagenesis to glutamine and expressed and analyzed the functional properties of the mutant channel. The affinity for tetrodotoxin was reduced over 10,000-fold. This amino acid residue is located in segment SS2 (Figs. 2A and 4) on the extracellular side of the S6 transmembrane segment in domain I of the sodium channel. Subsequent extension of their analysis identified acidic amino acid residues in the same position as glu387 in each domain, which were all required for high-affinity tetrodotoxin binding (Terlau *et al.*, 1991). These residues are therefore likely to surround the extracellular opening of the pore and contribute to a receptor site for tetrodotoxin. In addition to this ring of carboxyl residues, a second ring of amino acids located three residues on the amino terminal side of these is also required for tetrodotoxin binding (circles in Figs. 2A and 4). These are acidic amino acids in domains I and II, basic in domain III, and neutral in domain IV. If this region is in alpha helical conformation, this second set of residues required for tetrodotoxin binding would fall on the same side of the helix and form a second inner ring of residues at the opening of the pore.

Cardiac sodium channels bind tetrodotoxin with 200-fold lower affinity than brain or skeletal muscle sodium channels but retain all of the eight residues described above that are required for high-affinity binding. However, at position 385, two residues toward

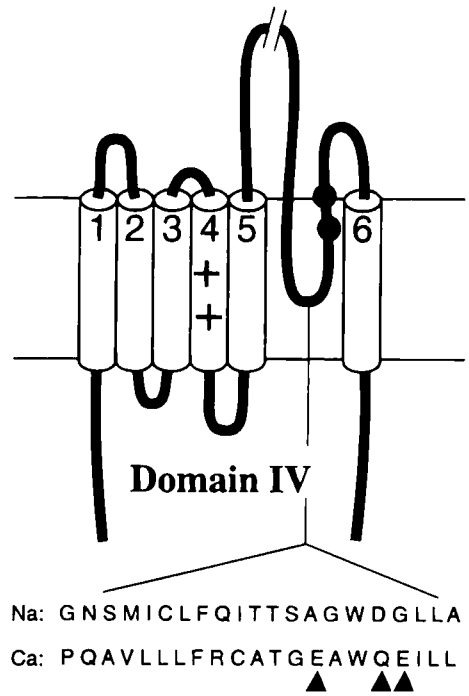

Fig. 4. Amino acid residues required for pore formation. The positions of amino acid residues required for ion conductance and selectivity are illustrated. Dark circles indicate positions of residues which determine the ion selectivity of sodium and calcium channels. The amino acid sequences of the SS1/SS2 region of the Type IIA sodium channel and the L-type skeletal muscle calcium channel are presented below. The arrow heads below each sequence illustrate the positions at which residues required for ion conductance and selectivity and for high affinity binding of the extracellular pore blocker tetrodotoxin are located.

the N-terminal from glu387 in the brain sodium channel II sequence, there is a change of a tyrosine or phenylalanine in the brain and skeletal muscle channels to cysteine in the cardiac sodium channel. Mutation of this residue from cys to phe or tyr causes an increase of 200-fold in the cardiac sodium channel, and the converse mutation causes a loss of affinity of 200-fold in the brain or skeletal muscle channel (Satin *et al.*, 1992; Backx *et al.*, 1992; Heinemann *et al.*, 1992a). Thus, it is likely that this residue also contributes in an essential way to the tetrodotoxin receptor site. Cadmium is a high-affinity blocker of cardiac sodium channels but not of brain or skeletal muscle sodium channels. Substitution of this critical cysteine in the skeletal muscle or brain sodium channel confers high-affinity block by cadmium on these channels (Satin *et al.*, 1992; Backx *et al.*, 1992). Analysis of the voltage dependence of cadmium block suggests that this ion passes 20% of the way through the membrane electrical

field in reaching its binding site formed by this cysteine residue (Backx *et al.*, 1992). Thus, this residue may be approximately 20% of the way through the electrical field within the pore of the sodium channel.

The Intracellular Mouth of the Pore

Local anesthetics and related antiarrhythmic drugs are thought to bind to a receptor site on the sodium channel which is accessible only from the intracellular side of the membrane and is more accessible when the sodium channel is open (Hille, 1992). Similarly, the phenylalkylamine class of calcium channel antagonists are characterized as intracellular open-channel blockers, and tetraethylammonium and related monoalkyl-trimethylammonium derivatives can block potassium channels from the intracellular side of the membrane when the channel is open (Hille, 1992). Both biochemical and molecular biological approaches have been used to probe the peptide segments of the principal subunits of the voltage-gated ion channels which interact with these intracellular pore blockers.

Verapamil and related phenylalkylamine calcium channel antagonist drugs are the highest-affinity ligands among the diverse intracellular pore blockers. Desmethoxyverapamil and its photoreactive azido derivative ludopamil have K_d's for equilibrium binding to purified calcium channels in the range of 30–50 nM and therefore can be used as highly specific binding probes of their receptor site in the intracellular mouth of the calcium channel. Covalent labeling of purified calcium channels with ludopamil results in incorporation into the $\alpha 1$ subunit only (Striessnig *et al.*, 1990). The site of covalent labeling was located by extensive proteolytic cleavage of the labeled $\alpha 1$ subunit followed by identification of the photolabeled fragments by immunoprecipitation with site-directed anti-peptide antibodies (Striessnig *et al.*, 1990). All of the covalent label recovered was incorporated into a peptide fragment containing the S6 segment of domain IV of the $\alpha 1$ subunit and several amino acid residues at the intracellular end of this transmembrane segment. Since phenylalkylamines act only from the inside of the cell, it was concluded that the intracellular half of transmembrane segment IVS6 and the adjacent intracellular residues form part of the receptor site for phenylalkylamines and therefore part of the intracellular mouth of the calcium channel (Striessnig *et al.*, 1990; Catterall and Striessnig, 1992).

A mutagenesis scan of the IVS6 segment of the cardiac calcium channel substituting alanine for the native residues in each position of the transmembrane segment identified three amino acid residues which are required for high-affinity binding of the phenylalkylamine pore blockers (Hockerman *et al.*, 1995). Residues Y1463, A1467, and I1470 are approximately aligned along one face of the putative transmembrane alpha helix (Fig. 5B). Mutation of each of them individually causes a 5- to 12-fold increase in the K_d for binding and block of calcium channels (Hockerman *et al.*, 1995). Mutation of all three residues causes a 100-fold reduction in the apparent affinity for phenylalkylamines, increasing the K_d for desmethoxyverapamil from 50 nM to 5 μM. Phenylalkylamines will block other types of calcium channels, sodium channels, and potassium channels at concentrations in the range of 5 μM, so it is likely that the unique high-affinity binding of these drugs to L-type calcium channels depends on interactions with these three amino acid residues. These residues may form part of a larger receptor site within the pore of the L-type calcium channel to which phenylalkylamines bind and block ion conductance.

Local anesthetics which block Na^+ channels also interact with amino acid residues in S6 segments. Analysis of the S6 segment in domain IV of the Na^+ channel α subunit by alanine-scanning mutagenesis revealed two aromatic amino acid residues in positions 1764 and 1771 which were required for high-affinity binding of a local anesthetic (Ragsdale *et al.*, 1994). These two residues are located approximately 11 Å apart on the same face of the proposed IVS6 α helix and may interact with the aromatic and positively charged amino groups of the drug molecule which are spaced 10 to 15 Å apart. It is likely that these pore-blocking drugs also interact with amino acid residues in the S6 segment of other domains of the Na^+ channel as they bind within the pore surrounded by all four domains.

The mutagenesis scan of the IVS6 segment of the sodium channel also revealed amino acid residues which restrict the movement of pore-blocking drugs through the extracellular mouth of the pore. Quaternary derivatives of local anesthetics, which are permanently charged and therefore membrane-impermeant, are effective blockers of brain sodium channels only when they are prefused or injected inside the cell (Hille, 1992). These results indicate that the receptor site for these drugs must be approached from the intracellular side of the membrane. Conversion of isoleucine 1760 near the extracellular end of the IVS6 segments to the

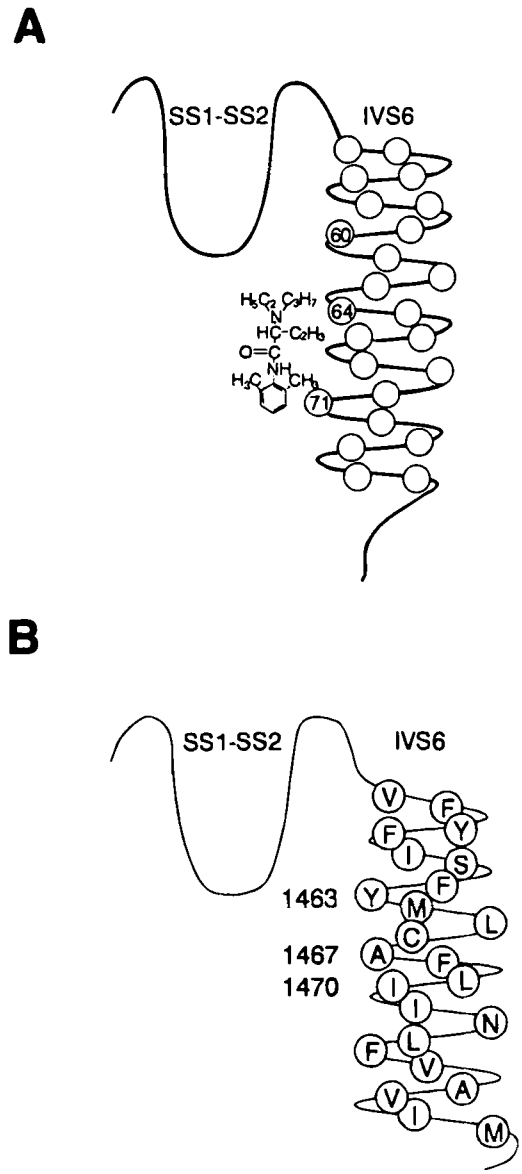

Fig. 5. Amino acid residues in transmembrane segments IVS6 that are required for high affinity binding of intracellular pore blockers of sodium and calcium channels. A. The local anesthetic etidocaine is depicted in its receptor site interacting with phe 1764 (64) and tyr 1771 (71) in segment IVS6 of the brain sodium channel. Ile 1760 (60) forms the extracellular limit of the receptor site. B. The amino acid residues of segment IVS6 of the α1 subunit of skeletal muscle calcium channels are indicted in single letter code and the three amino acid residues that are required for high affinity binding of phenylalkylamines are indicated by their sequence numbers; tyr 1463, ala 1467, and ile 1470.

smaller residues alanine creates a pathway through which QX314 can exit and enter the local anesthetic receptor site from the extracellular side of the membrane. Presumably this entry and exit requires movement through the outer mouth of the pore.

In contrast to brain sodium channels, cardiac sodium channels can be blocked by quaternary local anesthetics like QX314 from the extracellular side of the membrane without opening the channel (Alpert *et al.*, 1989). Conversion of threonine 1755 near the extracellular end of the IVS6 segment in the cardiac sodium channel to valine, its counterpart in the brain sodium channel, restricts extracellular access of QX314 (Qu *et al.*, 1995). Evidently, this molecular difference between brain and cardiac channels is sufficient to control QX314 access. Movement of QX314 through this access pathway is prevented by binding of tetrodotoxin to its receptor site in the extracellular mouth of the pore. Moreover, QX314 approaching from either the extracellular or intracellular side of the membrane binds to the same receptor site since mutation of phenylalanine 1762 in the cardiac sodium channels, the counterpart of phenylalanine 1764 in the brain channel, prevents binding of drug from either side of the membrane (Qu *et al.*, 1995). These results confirm that the drug molecule must move through the extracellular mouth of the pore in order to reach the same receptor site within the pore that is reached from the intracellular side of the membrane.

Ion Conductance and Selectivity

Consistent with the idea that the amino acid residues required for binding of pore blockers are also required for interaction with permeant ions, changes in these residues have dramatic effects on ion conductance and selectivity. A clear demonstation of the close relationship between the amino acid residues which determine pore blocking properties and those that determine ion conductance and selectivity came from analysis of the effects on ion selectivity of mutations which prevent binding of the pore blocker tetrodotoxin (Terlau *et al.*, 1991; Heinemann *et al.*, 1992b). Changes in the amino acid residues in the SS1/SS2 region that are important for binding of tetrodotoxin are also critical determinants of sodium channel ion conductance and selectivity. Single-channel conductance values for mutations which neutralize single charges among the six negatively charged amino acid residues that are important for tetrodotoxin binding also reduce single channel conductance, in some cases to as little as 10% of wild-type levels (Terlau *et al.*, 1991). Sodium and calcium channels have similar overall structures, but strikingly different ion selectivity. Sodium ions are essentially impermeant through calcium channels in the presence of calcium, but are rapidly permeant in

the absence of divalent cations (Hille, 1992). This property is thought to arise from high-affinity binding of calcium ions to two sites in the ion conductance pathway which blocks sodium ion entry and allows rapid calcium conductance. Calcium ions are less than 10% as permeable as sodium ions through the sodium channel. Remarkably, mutation of only two amino acid residues in the SS2 segments of domains III and IV (Fig. 2) sodium channel is sufficient to confer calcium channel-like permeability properties (Heinemann *et al.*, 1992b). Mutation of lysine 1422 and alanine 1714 to negatively charged glutamate residues not only altered tetrodotoxin binding but also caused a dramatic change in the ion selectivity of the sodium channel from sodium-selective to calcium-selective. In addition, these changes created a high-affinity site for calcium binding and block of monovalent ion conductance through the sodium channel, as has been previously described for calcium channels. Thus, in the mutant sodium channel with two additional negative charges near the extracellular mouth of the putative pore region, monovalent cation conductance is high in the absence of calcium. At calcium concentrations in the 10 μM range, monovalent cation conductance is strongly inhibited by high-affinity calcium binding. As calcium concentrations are increased, calcium conductance is preferred over sodium conductance. These results mirror the ion conductance properties of calcium channels and indicate that a key structural determinant of the ion selectivity difference between calcium and sodium channels is specified by the negatively charged amino acid residues at the mouth of the putative pore-forming region.

These results on Na^+ channels imply that the corresponding amino acid residues in Ca^{2+} channels are also important determinants of their ion selectivity. Consistent with this expectation, mutation of the corresponding amino acid residues in calcium channels does cause marked alterations in ion selectivity, ion binding, and block of the pore by divalent cations (Yang *et al.*, 1993; Tang *et al.*, 1993). These results demonstrate the importance of amino acid residues in analogous positions within the pore region in defining the selectivity of Na^+ and Ca^{2+} channels.

INACTIVATION

Molecular Mechanism of Sodium Channel Inactivation

The ion conductance activity of the sodium channels is terminated within a few milliseconds after opening by the process of inactivation. This is a critical feature of sodium channel function because persistent opening of the sodium channels would depolarize excitable cells permanently and kill them. Persistent activation of even a small fraction of sodium channels by neurotoxins is sufficient to cause continuing arrhythmias in the heart and repetitive action potential generation in neuronal preparations. Inactivation of sodium channels is at least 10-fold more rapid than the most rapid inactivation processes of calcium and potassium channels, and specific structural features of the sodium channel have evolved to mediate this rapid inactivation process.

Fast inactivation of the sodium channel acquires most of its voltage dependence from coupling to voltage-dependent activation, and the inactivation process can be specifically prevented by treatment of the intracellular surface of the sodium channel with proteolytic enzymes (Armstrong, 1981). These results led to the proposal of an autoinhibitory, "ball-and-chain" model for sodium channel inactivation in which an inactivation particle tethered on the intracellular surface of the sodium channel (the ball) diffuses to a receptor site in the intracellular mouth of the pore, binds, and blocks the pore during the process of inactivation (Armstrong, 1981). This model predicts that an inactivation gate on the intracellular surface of the sodium channel may be responsible for its rapid inactivation.

The sodium channel segments that are required for fast inactivation have been identified by use of a panel of site-directed anti-peptide antibodies against peptides corresponding to short (15–20 residue) segments of the α subunit. These anti-peptide antibodies were applied to the intracellular surface of the sodium channel from the recording pipet in whole cell voltage clamp experiments or from the bathing solution in single-channel recording experiments in excised, inside-out membrane patches (Vassilev *et al.*, 1988, 1989). In both cases, only one antibody, directed against the short intracellular segment connecting homologous domains III and IV (Figs. 2 and 6), inhibited sodium channel inactivation. Inhibition of fast inactivation of antibody-modified sodium channels in membrane patches was complete. The binding and effect of the antibody were voltage-dependent. At negative membrane potentials where sodium channels are not inactivated, the antibody bound rapidly and inhibited channel inactivation; at more positive membrane potentials where the sodium channel is inactivated, antibody binding and action were greatly slowed or prevented. Based on these results, it was proposed that the segment which this antibody recognizes is directly

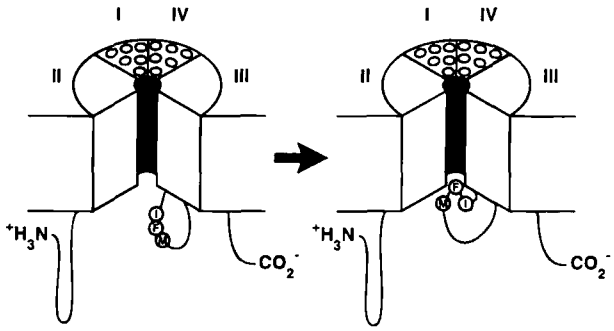

Fig. 6. Mechanism of inactivation of sodium channels. The hinged-lid mechanism of sodium channel inactivation is illustrated. The intracellular loop connecting domains III and IV of the sodium channel is depicted as forming a hinged lid. The critical residue phe1489 is shown as occluding the intracellular mouth of the pore.

involved in the conformational change leading to channel inactivation. During this conformation change, this inactivation gating segment was proposed to fold into the channel structure, serve as the inactivation gate by occluding the transmembrane pore, and become inaccessible to antibody binding (Vassilev *et al.*, 1988, 1989) (Fig. 6).

A similar model is supported by site-directed mutagenesis experiments (Stühmer *et al.*, 1989). Expression of the sodium channel α subunit in *Xenopus* oocytes as two pieces corresponding to the first three domains and the fourth domain results in channels that activate normally but inactivate 20-fold more slowly than normal. The physiological characteristics of these cut channels are similar to those of sodium channels with inactivation blocked by the site-directed antibody. In contrast, sodium channel α subunits cut between domains III and IV have normal functional properties. These two independent approaches using site-directed antibodies and cut mutations provide strong support for identification of the short intracellular segment connecting domains III and IV as an inactivation gating loop.

The inactivation gating loop contains highly conserved clusters of positively charged and hydrophobic amino acid residues. Neutralization of the positively charged amino acid residues in the inactivation gating loop of the sodium channel by site-directed mutagenesis does not have a profound effect on channel inactivation (Moorman *et al.*, 1990; Patton *et al.*, 1992), although neutralization of the cluster of positively charged residues at the amino terminal end of the loop does slow inactivation and shift the voltage dependence of both activation and inactivation (Patton *et al.*, 1992). In contrast, deletion of the 10-amino acid segment at the amino terminal end of the loop completely blocks fast sodium channel inactivation (Patton *et al.*, 1992). Scans of the hydrophobic amino acid residues in this segment of the inactivation gating loop by mutation to the hydrophilic, but uncharged residue glutamine shows that mutation of the three-residue cluster IFM to glutamine completely blocks fast sodium channel inactivation (West *et al.*, 1992). The single phenylalanine in the center of this cluster at position 1489 in sodium channel II is the critical residue (Fig. 6). Conversion of this residue to glutamine is sufficient by itself to nearly completely prevent fast channel inactivation. Mutation of the adjacent isoleucine and methionine residues to glutamine also has substantial effects. Substitution of glutamine for isoleucine slows inactivation 2-fold and makes inactivation incomplete, leaving 10% sustained current at the end of long depolarizations. Substitution of glutamine for methionine slows inactivation 3-fold. The interaction of phe1489 with the receptor of the inactivation gating particle is likely to be hydrophobic because there is a close correlation between the hydrophobicity of the residue at that position and the extent of fast sodium channel inactivation (Scheuer *et al.*, 1993). On the basis of these results, it has been proposed that these residues serve as the inactivation gating particle entering the intracellular mouth of the transmembrane pore of the sodium channel and blocking it during channel inactivation. The intracellular loop between domains III and IV therefore serves as an inactivation gate and closes the transmembrane pore of the sodium channel from the intracellular side of the membrane.

This model for inactivation implies that the intracellular mouth of the pore has a receptor site that binds the inactivation particle. In agreement with this, a pentapeptide containing the IFM motif restores inactivation to mutant Na^+ channels with phe 1489 replaced by glutamine (Eaholtz *et al.*, 1994). The freely diffusible peptide can restore inactivation with the rapid kinetics and steep voltage dependence of the native Na^+ channel. Alteration of the IFM motif by substitution of glutamine or alanine residues prevents restoration of inactivation, consistent with the conclusion that the pentapeptide does indeed bind to a receptor which requires the same structure as the inactivation particle itself.

A candidate for the inactivation gate receptor region was identified by alanine-scanning mutagenesis of the S6 segment in domain IV (McPhee *et al.*, 1994). Mutation of three adjacent hydrophobic residues at the intracellular end of this transmembrane segment to

alanine produces Na^+ channels which inactivate incompletely having >85% of their Na^+ current remaining at the end of 15-ms depolarizing pulses. This mutation also causes prolonged single-channel openings and frequent reopenings that are characteristic of Na^+ channels whose fast inactivation process has been disrupted. However, further analysis revealed that fast inactivation of this mutant was still restored by a peptide containing the IFM motif. Thus, the receptor for the IFM motif is intact in this mutant and the residues at the intracellular end of segment IVS6 must be involved in the inactivation process in another unexpected way that is required for the stability of the inactivated state.

A Hinged-Lid Model of Sodium Channel Fast Inactivation

The structure of the sodium channel inactivation gate does not fit the expectations of the "ball-and-chain" model of inactivation very well. The inactivation gate is evidently formed by a short loop of the channel polypeptide which is predicted to be highly structured (Guy and Seetharamulu, 1986). It places the inactivation particle close to the mouth of the transmembrane pore. The inactivation gate loop resembles more closely the "hinged lids" of allosteric enzymes which are rigid peptide loops that fold over enzyme active sites and control substrate access. Conformational changes induced by the binding of allosteric ligands move the hinged lid away from the active site and allow substrate access and catalytic activity. In analogy to the allosteric enzymes, it has been proposed that the sodium channel inactivation gate functions as a hinged lid which pivots to place the inactivation particle in a position to bind to the intracellular mouth of the transmembrane pore of the sodium channel (West *et al.*, 1992) (Fig. 6). The three-dimensional structures of some hinged lids of allosteric enzymes which are known from X-ray crystallographic and two-dimensional NMR studies provide a valuable structural model for design of further experiments to define the mechanism of sodium channel inactivation in more detail.

Inactivation of Calcium Channels

Calcium channels inactivate by different mechanisms. Both voltage-dependent inactivation mechanisms, like the sodium channel, and calcium-dependent mechanisms, which are unique for the calcium channel, have been identified at the cellular level. Voltage-dependent inactivation of most calcium channels is much slower than sodium channels, with time constants for half inactivation ranging from 25 to several hundred msec. The intracellular loop connecting domains III and IV of calcium channels is not closely related in amino acid sequence to the inactivation gating loop of sodium channels, and no evidence has been presented so far to implicate this region of the calcium channel in voltage-dependent inactivation. The difference in the rate of inactivation of two distinct calcium channels has been traced to transmembrane segment S6 in domain I by analysis of chimeric $\alpha 1$ subunits (Zhang *et al.*, 1994). The mechanism through which this segment influences channel inactivation is unknown at present.

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