Annu. Rev. Biochem. 1995. 64:493–531

STRUCTURE AND FUNCTION OF VOLTAGE-GATED ION CHANNELS

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KEY WORDS: Ion channel, action potential, electrical excitability, ion transport, membrane proteins

CONTENTS

0066-4154/95/0701-0493$00.00

ABSTRACT

Voltage-gated ion channels are responsible for generation of 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 that 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 this family of channels is built upon a common structural theme with variations appropriate for functional specialization of each channel type.

INTRODUCTION

The voltage-gated sodium, calcium, and potassium channels are responsible for the generation of conducted electrical signals in neurons and other excitable cells. The permeability increase resulting from activation of these channels is biphasic. Upon depolarization, permeability to sodium, calcium, or potassium increases dramatically over a period of 0.5 to hundreds of msec and then decreases to the baseline level over a period of 2 msec to a few seconds. This biphasic behavior results from two experimentally separable gating processes that control ion channel function: activation, which controls the rate and voltage dependence of the permeability increase following depolarization, and inactivation, which controls the rate and voltage dependence of the subsequent return of the ion permeability to the resting level during a maintained depolarization. The voltage-gated ion channels can therefore exist in three functionally distinct states or groups of states: resting, active, and inactivated. Both resting and inactivated states are nonconducting, but channels that have been inactivated by prolonged depolarization are refractory unless the cell is repolarized to allow them to return to the resting state. The ion conductance of the activated ion channels is both highly selective and remarkably efficient. Selectivity among the physiological ions ranges from 12-fold for sodium channels to over 1000-fold for calcium channels, and all three classes of ion channels conduct ions across biological membranes at rates approaching their rates of free diffusion through solution. Understanding the molecular bases for voltage-dependent activation, rapid inactivation, and selective and efficient ion conductance is a major goal of current research on these critical signaling proteins.

STRUCTURE AND FUNCTION OF SODIUM CHANNEL SUBUNITS

Purification and Characterization

The initial determination of the subunit structure of the rat brain Na^+ channel took advantage of neurotoxins that bind with high affinity and specificity to the channel complex and thus could be used as molecular probes to identify its protein components. Five groups of neurotoxins that act at different receptor sites on the Na^+ channel have been described (1–3). Briefly site 1 binds tetrodotoxin, saxitoxin and μ-conotoxin which block ion conductance. Site 2 binds the toxins, batrachotoxin, veratridine, grayanotoxin, and aconitine, resulting in persistent activation of the Na^+ channel. Site 3 binds the polypeptide α-scorpion toxins and sea anemone toxins which slow or block inactivation. Agents which bind at this site also enhance the persistent activation of the Na^+ channel caused by toxins acting at neurotoxin receptor site 2. Receptor site 4 binds a second class of scorpion toxins (β-scorpion toxins) that shift the voltage dependence of activation to more negative membrane potentials without modifying Na^+ channel inactivation. Finally, receptor site 5 binds the brevetoxins and ciguatoxins, agents that cause repetitive neuronal firing, shift the voltage dependence of Na^+ channel activation, and block Na^+ channel inactivation.

Direct chemical identification of the 260 kD α subunit and the 36 kD β1 subunit of the rat brain sodium channel in situ was accomplished by specific covalent labeling of neurotoxin receptor sites 3 and 4 on the Na^+ channel complex with photoreactive derivatives of α- and β-scorpion toxins, respectively (4–6). Separation of two photoreactive derivatives of an α-scorpion toxin by ion-exchange chromatography allowed selective labeling of each subunit (7). Radiation inactivation studies were also used to probe the molecular sizes of the subunits of the Na^+ channel. Measurements of the target size for inactivation of either tetrodotoxin or α-scorpion toxin binding to the Na^+ channel from rat brain or eel electroplax revealed a structure of 230 kD to 266 kD (8, 9). In contrast, radiation inactivation studies of β-scorpion toxin binding activity of rat brain sodium channels implicated two polypeptides of 266 kD and 45 kD, consistent with a role of both α and β1 subunits in formation of neurotoxin receptor site 4 (10).

The sodium channel from electric eel electroplax was purified using the binding of radiolabeled tetrodotoxin as a specific assay (11). It consisted of a single polypeptide of 280 kD, similar in size to the α subunit of the rat brain sodium channel (12), with a high level of N-linked carbohydrate (13).

Purification of the intact sodium channel from rat brain using high affinity binding of saxitoxin as an assay revealed a complex of one α subunit of 260 kD and two distinct β subunits: β1 with an apparent molecular mass of 36 kD,

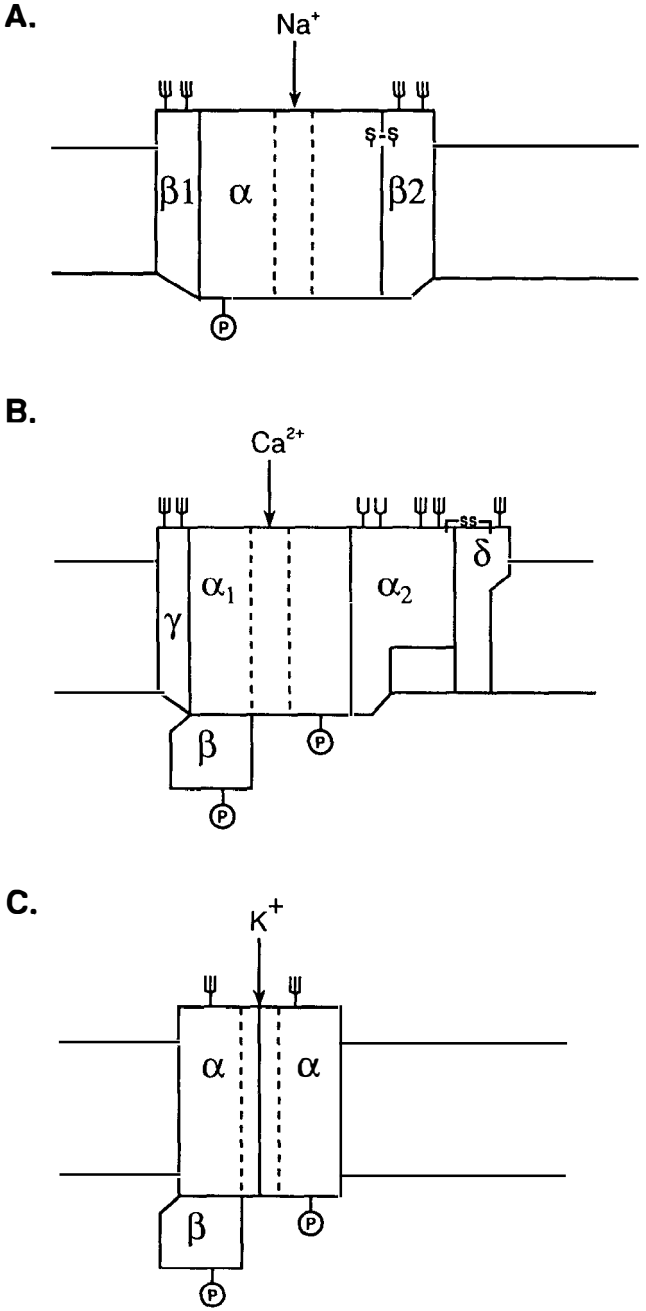
and β2 with an apparent molecular mass of 33 kD (14–17). The β2 subunit is covalently attached to the α subunit by disulfide linkage while the β1 subunit is noncovalently associated. The subunit stoichiometry of 1:1:1 yielded a molecular weight (329 kD) in close agreement with that of the solubilized oligomeric channel (316 kD) (18). Partial proteolytic maps showed that the β1 and β2 subunits were distinct and probably unrelated (17). Both the β1 and β2 subunits were covalently labeled by a hydrophobic probe specific for transmembrane segments of proteins in mixed micelles of Triton X-100 and phosphatidylcholine or in reconstituted phosphatidylcholine vesicles, and both subunits were preferentially extracted into the nonionic detergent Triton X-114 in a phase separation procedure, as expected for intrinsic membrane proteins (19). The α subunits and both β subunits are heavily glycosylated (17, 20). The apparent molecular weights of the deglycosylated subunits were determined to be 220 kD, 23 kD, and 21 kD for α, β1 and β2, respectively, which suggests that a substantial fraction of the mass of the native subunits is carbohydrate and that much of the protein mass is exposed on the extracellular surface (17). These experiments led to a heterotrimeric model for the subunit structure of the brain sodium channel as illustrated in Figure 1A.

The Na^+ channel from rat and rabbit skeletal muscle sarcolemma contains a large α subunit of 260 kD and a small β subunit of approximately 38 kD (21–23). Enzymatic deglycosylation of the purified β subunit yielded a core peptide of 26.5 kD. Although the 38 kD subunit of the Na^+ channel could be resolved into a doublet of 37 kD and 39 kD, stoichiometric analysis suggested that there is only a single β subunit associated noncovalently with each large α subunit. The noncovalent association implies that the β subunit in purified skeletal muscle sodium channels is β1-like. Studies using affinity-purified polyclonal antibodies to the purified rat brain β1 subunit identified immunoreactive β1-like subunits in rat skeletal muscle that appeared as a closely spaced doublet of 41 and 38 kD on SDS-PAGE, consistent with the results obtained using purified sodium channels (24).

Cloning and Primary Structure of the Sodium Channel α Subunit

Cloning of the α subunit of the sodium channel from eel electroplax by Noda, Numa, and colleagues (25) gave the initial insight into the primary structure of a voltage-gated ion channel. Using oligonucleotides encoding short segments of the electric eel electroplax sodium channel and antibodies directed

Figure 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.



against it, Noda et al (25) isolated cDNAs encoding the entire polypeptide from expression libraries of electroplax mRNA. The deduced amino acid sequence revealed a protein with four internally homologous domains, each containing multiple potential alpha-helical transmembrane segments (Figure 2A). The wealth of information contained in this deduced primary structure has revolutionized research on the voltage-gated ion channels.

The cDNAs encoding the electroplax sodium channel were used to isolate cDNAs encoding three distinct, but highly homologous, rat brain sodium channels (types I, II, and III) (26, 27). cDNAs encoding the alternatively spliced type IIA sodium channel were isolated independently by screening expression libraries with antibodies against the rat brain sodium channel α subunit (28, 29). The type II gene contains two adjacent exons encoding segment IS3 (30) that are alternatively spliced into mature mRNA in a developmentally regulated manner. The type II is most prominent in embryonic and neonatal brain while the type IIA form is most prominent in the adult brain (30, 31). cDNAs encoding the type II/IIA sodium channel were used as probes to isolate cDNAs encoding sodium channel α subunits expressed in skeletal muscle and heart by low-stringency hybridization (32–34). The μ1 sodium channel α subunit is expressed primarily in adult skeletal muscle (32); the h1 sodium channel α subunit is expressed primarily in heart and also in uninnervated or denervated skeletal muscle (33, 34). These sodium channels have a close structural relationship to the three brain sodium channel α subunits. In general, the similarity in amino acid sequence is greatest in the homologous domains from transmembrane segment S1 through S6, while the intracellular connecting loops are not highly conserved.

Complementary DNAs encoding α subunits of three distinct sodium channels from *Drosophila* have been cloned by cross-hybridization, and most of the primary structures of the corresponding sodium channels have been deduced (35–37). Thus, it appears that *Drosophila* also has multiple sodium-channel genes as observed in rat. Presumably these distinct genes have distinct roles in electrical excitability in both species.

More recently, new putative sodium channel α subunits have been cloned from glial and heart cDNA libraries (38, 39). The amino acid sequences retain the four-domain structure of the other α subunits and many of their other conserved features, but are distinctly more divergent than the other α subunits previously characterized. It has been suggested that these new glial/heart sodium channels define a new sub-family of sodium channels.

Cloning and Primary Structure of the β1 and β2 Subunits

cDNA clones encoding the β1 subunit of the rat brain Na^+ channel were isolated using a combination of polymerase chain reaction and library-screening techniques based on the amino acid sequence of the amino terminus of the

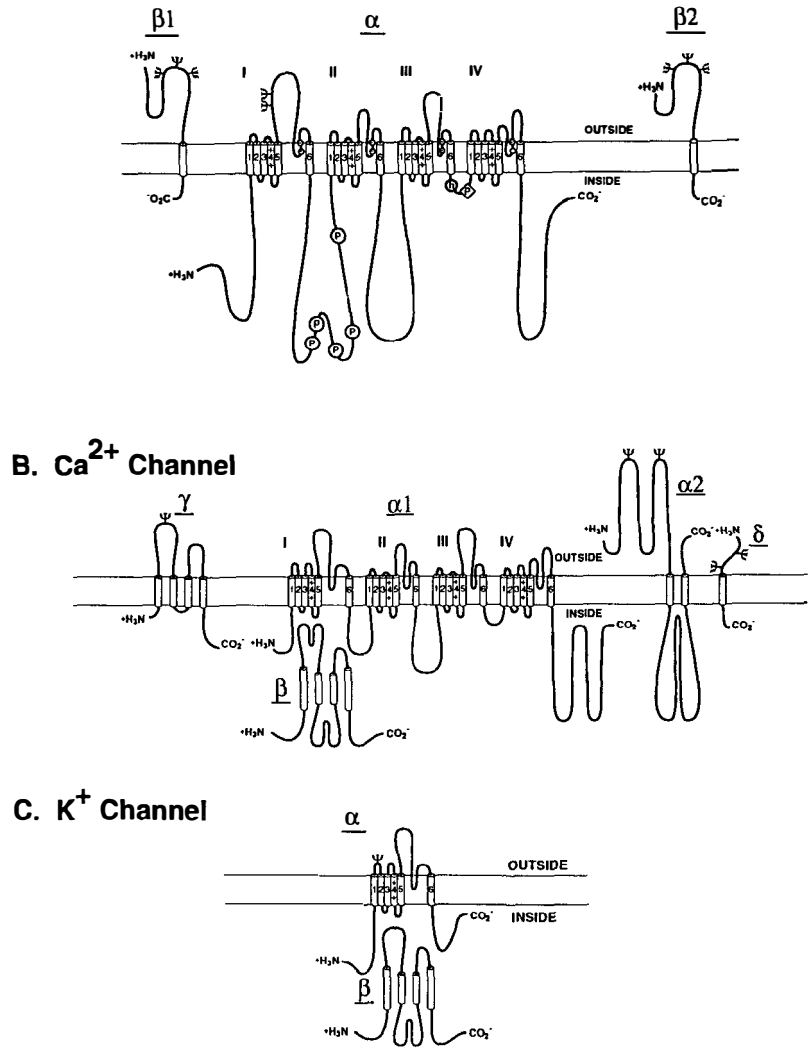


Figure 2 Transmembrane organization of the ion channel subunits. The primary structures of the subunits of the voltage-gated ion channels are illustrated. Cylinders represent probable alpha helical segments. *Bold lines* represent the polypeptide chains of each subunit with length approximately proportional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

purified protein (40). The deduced primary structure indicates that the β1 subunit is a 22,851-dalton protein that contains a small cytoplasmic domain, a single putative transmembrane segment, and a large extracellular domain with four potential N-linked glycosylation sites (Figure 2A), consistent with previous biochemical data (17, 19). Northern blot analysis revealed a 1400-nucleotide mRNA in rat brain, heart, and spinal cord, and at low levels in rat skeletal muscle.

A similar approach was taken to cloning the β2 subunit. It also has a single transmembrane segment, a small intracellular carboxyl terminal domain, and a large glycosylated extracellular amino-terminal domain (unpublished results).

Functional Role of Na^+ Channel Subunits

Early biochemical experiments pointed to a dominant functional role for the α subunits. Covalent labeling and radiation-inactivation studies implicated α subunits in formation of the receptor sites for tetrodotoxin and saxitoxin (site 1) (8) and both α- and β-scorpion toxins (sites 3 and 4) (4, 5, 7). In addition, Na^+ channels purified from eel electroplax and chicken heart contained only α subunits but retained high affinity for tetrodotoxin and, in the case of eel electroplax, ion-conductance activity (12, 41, 42). Because tetrodotoxin and saxitoxin are thought to block the pore of the Na^+ channels and the scorpion toxins affect activation and inactivation gating, these biochemical results suggested that α subunits were involved in both ion conductance and gating.

Potential functional roles of the β1 and β2 subunits were analyzed in purified and reconstituted sodium channel preparations. Selective removal of the β1 subunit from the αβ2 complex of detergent-solubilized or reconstituted rat brain Na^+ channels resulted in the complete loss of [^{3}H]saxitoxin-binding activity, veratridine-activated $^{22}Na^+$ influx, α-scorpion toxin binding activity, and voltage-activated ion conductance (43, 44). Tetrodotoxin quantitatively stabilized the solubilized complex against the loss of the β1 subunit and loss of functional activities. In contrast, removal of the β2 subunit by reduction of disulfide bonds yielded a preparation of αβ1 that retained Na^+ channel functions. These studies showed that a complex of α and β1 subunits is both necessary and sufficient for channel function in the purified state and suggested that β1 subunits, but not β2 subunits, are required to stabilize the functional state of purified brain sodium channels in detergent solution.

The role of the α and β1 subunits in Na^+ channel function in an intact cell was tested directly through expression of the α and β1 subunits in *Xenopus* oocytes. RNA encoding α subunits from brain, skeletal muscle, and heart alone is sufficient to encode functional Na^+ channels in *Xenopus* oocytes (28, 29, 32, 45–48). However, their inactivation is slow relative to that observed in neurons or skeletal muscle, and their voltage dependence of inactivation is

shifted to more positive membrane potentials (29, 49–51). Co-expression of low-molecular-weight RNA from rat brain or skeletal muscle accelerates inactivation, shifts the voltage dependence of inactivation to more negative membrane potentials, and increases the peak amplitude of Na^+ current expressed from cloned α subunits (29, 32, 50, 51). These results suggested a possible role for the low-molecular-weight β1 and/or β2 subunits in Na^+ channel function. Co-expression of type IIA α subunits and β1 subunits in *Xenopus* oocytes resulted in a 2.5-fold increase in the amplitude of the peak Na^+ current, an increase in the rate of activation, a 5-fold acceleration in the rate of inactivation, and a 19 mV shift in the hyperpolarizing direction of steady state fast inactivation (40, 52). Co-expression of rat or human β1 subunits with the rat skeletal muscle μ1 α subunit or rat brain type III α subunits in *Xenopus* oocytes gave similar results (52–54).

Na^+ channel α subunits have also been expressed both stably and transiently in mammalian cells in culture (55–57). Stable lines of Chinese hamster ovary cells expressing only the type IIA rat brain Na^+ channel α subunit generate Na^+ currents with a normal time course (55, 56), in spite of the lack of β1 subunits detectable by Northern blot, Western blot, and activity assays in *Xenopus* oocytes (58). However, the level of functional Na^+ channel expression in these cell lines is low relative to mRNA levels. Co-expression of β1 subunits with type IIA α subunits in mammalian cell lines results in an increase in the peak Na^+ current and the total number of Na^+ channels detected by saxitoxin binding, as well as a shift in the voltage dependence of activation and inactivation (58). Thus, β1 subunits in mammalian cells may stabilize the Na^+ channel complex in the plasma membrane, which results in increased functional expression, and also may alter the voltage dependence of channel gating. Additional experiments are needed to address whether association of different β1 subunits with α subunits can modify the function of the α subunits.

Mechanism of Action of β1 Subunits

β1 subunits have multiple effects on sodium channel function—increased peak current, accelerated activation and inactivation, and altered voltage dependence of inactivation. These multiple effects may result from distinct molecular interactions between α and β1 subunits or from a single molecular mechanism that alters multiple aspects of sodium-channel function. Cloned skeletal muscle sodium channel α subunits and type IIA and III brain sodium channel α subunits expressed in *Xenopus* oocytes exhibit two prominent gating modes: a rapid mode in which inactivation is complete in a few milliseconds, and a slow gating mode in which inactivation is slow and single sodium channels open repeatedly during long depolarizations (51, 59, 60). The effects of β1 subunits on the time courses of activation and inactivation, and possibly effects on the voltage dependence of inactivation, appear to result from a shift from the slow to the rapid gating mode

induced by co-expression of the β1 subunit (52, 54). Thus, a single molecular interaction with the β1 subunit may be sufficient to change the energetic relationship between these two gating modes of the α subunits and cause a shift of sodium channels to the rapid gating mode. This shift in gating mode would affect the multiple aspects of sodium channel function that differ between the two functionally distinct modes of gating.

STRUCTURE AND FUNCTION OF CALCIUM CHANNEL SUBUNITS

Molecular Properties of the Subunits of Skeletal-Muscle Calcium Channels

The calcium channels in the transverse tubule membranes of skeletal muscle have served as a primary biochemical and molecular model for studies of calcium channels because of their abundance. These channels serve two critical physiological roles. Like other calcium channels, they mediate calcium entry in response to deploarization. However, the voltage-gated calcium channels in skeletal muscle activate very slowly, and the calcium entering vertebrate skeletal muscle through voltage-gated calcium channels is not required for muscle contraction. It appears to serve to replenish cellular calcium during periods of rapid activity and to increase intracellular calcium in response to tetanic stimulation, which leads to increased contractile force. The primary physiological role for the skeletal muscle calcium channel is to serve as a voltage sensor in excitation-contraction coupling. Voltage-gated calcium channels in the transverse tubule membranes are thought to interact physically with the calcium-release channels located in the sarcoplasmic reticulum membrane. Voltage-driven conformational changes in the voltage-gated calcium channels then activate the calcium release from the sarcoplasmic reticulum via protein-protein interactions (61–63).

The voltage-gated calcium channels in skeletal muscle are L-type (64–66). They mediate long-lasting currents with slow voltage-dependent inactivation, they have a large single-channel conductance (about 25 pS) and a high voltage of activation, and they are specifically inhibited by dihydropyridine calcium-channel antagonists. The initial purification of the L-type calcium channels from skeletal muscle took advantage of their high density in transverse tubule membranes to provide an enriched starting material and employed the specific, high-affinity binding of dihydropyridine calcium channel antagonists to identify the calcium channel protein (67, 68). A heterogeous α subunit band (67, 68) and associated β subunits of 50 kD and γ subunits of 33 kD (67) were identified as components of the calcium channel in the initial purification studies as assessed by comigration during column chromatography and su-

crose-gradient sedimentation. Subsequent experiments demonstrated that the heterogenous α subunit band contained not only the principal α1 subunits with an apparent molecular mass of 175 kD but also a disulfide-linked dimer of α2 and δ subunits with apparent molecular masses of 143 kD and 27 kD, respectively (69–73). These results, together with analysis of the biochemical properties of the subunits, led to a model of calcium channel subunit structure illustrated in Figure 1B (2, 70). The specific association of these proteins as a multisubunit complex was supported by the copurification of each subunit with the dihydropyridine-binding activity and calcium-conductance activity of the calcium channel (67, 70, 74, 75), by co-immunoprecipitation of all these proteins by antibodies directed against the α1 subunits (69, 70, 76), and by co-immunoprecipitation of the calcium channel complex by antibodies against each auxiliary subunit (77–79). Estimates of stoichiometry indicated that each mol of calcium channel complex contains approximately 1 mol of each of the five subunits. The biochemical and molecular properties of each of the subunits of skeletal muscle calcium channels are considered below.

The α1 subunit of skeletal muscle calcium channels was cloned by library screening based on amino acid sequence (80). The cDNA predicts a protein of 1873 amino acids with a molecular weight of 212 kD, considerably larger than the estimate of 175 kD for the α1 subunits of purified calcium channels. Analysis of the α1 subunits of purified calcium channels and calcium channels in transverse tubule membranes using sequence-directed antibodies showed that most (more than 90%) were truncated in their carboxyl terminal domain between residues 1685 and 1699, which resulted in a 190-kD form that runs anomalously in SDS gels at 175 kD (81, 82). Only a small fraction (less than 10%) contained the full-length α1 subunit encoded by the cDNA. Both forms are detected in rat skeletal muscle cells in culture suggesting that both may be present in vivo (83). Since no mRNA that encodes the more abundant, truncated form has been identified, the truncated form may be produced by specific proteolytic processing in vivo.

The α2 subunit of skeletal muscle calcium channels is a hydrophobic glycoprotein with an apparent molecular mass of 143 kD before deglycosylation and 105 kD after deglycosylation (70, 84). It contains both high-mannose and complex carbohydrate chains. Cloning and sequencing cDNAs that encode the α2 subunit defined a protein of 1106 amino acids with a molecular mass of 125 kD, multiple potential transmembrane segments, and multiple consensus sites for N-linked glycosylation (85) (Figure 2B). The predicted α2 protein was 20 kD larger than the apparent molecular mass of the deglycosylated α2 subunit, which suggested that a portion of the protein encoded by the α2 cDNAs may not be present in the mature α2 subunit that had been characterized biochemically. Subsequent studies have shown that both α2 and δ subunits are encoded by the same mRNA (see below).

The β subunits are hydrophilic proteins that are not glycosylated and therefore are likely to be located on the intracellular side of the membrane (70). They are phosphorylated by multiple protein kinases, including protein kinase C and cAMP-dependent protein kinase, which regulate the function of many calcium channels (70, 75, 86–88). cDNA cloning and sequencing revealed a protein of 524 amino acids with a predicted molecular mass of 58 kD (89). In agreement with biochemical data, the primary structure does not include any potential transmembrane segments but contains multiple consensus sites for phosphorylation by different protein kinases (Fig. 2B).

The γ subunit of skeletal muscle calcium channels is a hydrophobic glycoprotein with an apparent molecular mass of 30 kD without deglycosylation and 20 kD following deglycosylation (70). Cloning and sequencing cDNAs encoding γ subunits revealed a protein of 222 amino acid residues with a molecular mass of 25 kD (90, 91). The deduced primary structure contained three predicted hydrophobic transmembrane segments and multiple sites for N-linked glycosylation.

The δ subunit appears on SDS gels as a doublet of 24 and 27 kD proteins, which are both hydrophobic and glycosylated (70, 92). Determination of the amino acid sequences of peptides derived from the δ subunit showed that it was encoded by the same mRNA as the α2 subunit (93, 94). The mature α2 subunit is truncated at alanine 934 of the α2δ precursor protein; residues 935–1106 constitute the disulfide-linked δ subunit. This sequence encodes a protein of 16 kD and contains a single transmembrane segment and three consensus sequences for N-linked glycosylation. The doublet on SDS gels represents two differently glycosylated forms of the δ subunit.

Subunits of Purified Cardiac Calcium Channels

Like the skeletal muscle calcium channels, the principal cardiac calcium channels are L-type (95, 96). Antibodies against α2δ subunits of skeletal muscle calcium channels detect corresponding subunits in cardiac preparations (97, 98). Partially purified cardiac calcium channels appear to contain α1, α2δ, and β subunits (99–103), but the relatively low abundance of calcium channels in cardiac tissue and the difficulty of controlling proteolysis during lengthy purification procedures have frustrated attempts at complete purification of an intact cardiac calcium channel complex. The α1 subunit with an apparent mass of 165 kD to 190 kD has been directly identified by photoaffinity labeling with photoreactive dihydropyridines (99, 100, 104).

Subunits of Purified Neuronal Calcium Channels

Multiple types of calcium channels, which differ in physiological and pharmacological properties, are expressed in neurons. At least three types of high-voltage-activated calcium channels have been distinguished in addition to L-type

(105–108). N-type, P-type, and Q-type channels all have intermediate single-channel conductances (about 15 pS) and can mediate calcium currents with varying rates of voltage-dependent inactivation, depending on their subunit composition (see below) and on other factors. They are best distinguished by their pharmacological properties: N-type are specifically inhibited by ω-conotoxin GVIA, P-type are most sensitive to ω-agatoxin IVA, and Q-type are most sensitive to ω-conotoxin MVIIC. Because the concentration of calcium channels in skeletal muscle transverse tubules is much higher than in neuronal membranes, the biochemical properties of these channels in neurons are not as well established. Immunoprecipitation with specific antibodies gainst α2δ subunits revealed a complex of polypeptides with sizes corresponding to α1, α2δ, and β subunits of dihydropyridine-sensitive L-type calcium channels in the brain (79, 109, 110). A novel 100 kD protein was also identified as a specifically associated component of the L-type calcium channel complex from brain (79). The ω-conotoxin-sensitive N-type calcium channels purified from rat brain contain an α1 subunit, a 140 kD α2-like subunit, and β subunits of 60 kDa to 70 kD, as identified by antibodies against the skeletal muscle forms of these subunits (111–115). Both L-type and N-type calcium channels from brain appear to lack a γ subunit, but proteins of approximately 100 kD are specifically associated with N-type calcium channels as well as L-type calcium channels from brain and may be additional, brain-specific subunits or associated proteins (79, 112, 114, 115).

Multiple Isoforms of the Subunits of Calcium Channels

Five additional genes encoding the α1 subunits of calcium channels have been identified by cDNA cloning and sequencing using the skeletal muscle α1 subunit as a probe, and these are thought to encode the major types of high-voltage-activated calcium channels defined by physiological and pharmacological properties (108, 116, 117). The α1 subunits fall into two groups based on amino acid sequence similarity. The class C and D genes encode L-type calcium channels in which the sequences are greater than 75% identical to skeletal muscle L-type α1 subunits. The class C gene is the primary calcium channel in the heart and is widely expressed in other tissues. The class D gene is expressed in neuroendocrine cells and neurons. The class A, B, and E genes encode non-L-type calcium channels, expressed primarily in neurons, in which the amino acid sequences are only 25 to 40% identical to the skeletal muscle α subunits. In general, the level of amino acid sequence identity among the α1 subunits is greatest in the transmembrane regions and least in the large intracellular loops connecting domains I, II, and III and in the intracellular amino-terminal and carboxy terminal domains. Most of the α1 subunit genes also encode alternatively spliced segments that increase their molecular diversity. The functional significance of alternative splicing has not yet been defined for any of these isoforms.

Four genes encoding calcium channel β subunits (β1–β4) have been characterized, and three of these have multiple splice products that have been identified (89, 118–123). In general, the amino acid sequences of the β subunits have two conserved segments in a central core region and are divergent in the carboxy and amino-terminal segments. All of the isoforms have consensus sequences for physphorylation by multiple protein kinases. Similar isoforms of the β subunit have been described in human tissues (124).

α2δ mRNAs that are recognized by hybridization at high stringency with cDNA probes encoding the skeletal muscle isoform are detected in total RNA from a wide range of tissues (85, 125). These α2δ primary transcripts are apparently the products of the same gene, but are differentially processed in human tissues to yield at least three isoforms which were designated by Williams et al (124) as $\alpha 2_a$ (expressed in skeletal muscle), $\alpha 2_b$ (expressed in neuronal tissues), and $\alpha 2_c$ (expressed in aorta) (124, 125).

In contrast to the α2δ and β subunits, there is no evidence to-date for multiple isoforms of the γ subunits. cDNA probes derived from the coding sequence of the γ subunit hybridize to a single mRNA species from skeletal muscle, but show weak or no specific hybridization in brain and several other tissues (90, 91, 126). Thus, the γ subunit may be encoded by a single gene which is expressed primarily or exclusively in skeletal muscle.

Functional Roles of Calcium Channel Subunits

Like the α subunits of Na^+ channels, the α1 subunits of some calcium channels can serve as voltage-gated ion channels when expressed alone. These include the L-type calcium channels from heart ($\alpha 1_{C1}$) (127) and skeletal muscle (128) and the class A and B α1 subunits from rat brain (129–131). However, the auxiliary subunits of calcium channels have substantial effects on expression and gating of the α1 subunits.

The α1 subunit of the skeletal muscle L-type calcium channel alone directs the synthesis of a low density of functional calcium channels in only a small fraction of the transfected cells in a mammalian L cell line (128). The currents expressed in L cells activated at least 10 times slower than calcium currents in skeletal muscle. However, the time courses of activation and inactivation of calcium currents in these cells were dramatically accelerated by co-expression of the β_{1a} subunit (132, 133). Co-expression of α2δ and γ subunits had little or no effect on the amplitude and kinetics of calcium currents of the skeletal muscle α1 subunits expressed in L cells (132). However, α2 subunits increase the activity of purified calcium channels reconstituted in lipid bilayers (134).

Expression of the smooth-muscle splice variant of the Class C α1 subunit in CHO cells also revealed substantial effects of auxiliary subunits (135). Peak calcium channel currents were increased, the kinetics of activation and inac-

tivation were accelerated, and the voltage dependence of activation and inactivation were shifted slightly to more negative membrane potentials. The $\alpha 2\delta$ subunit had further effects on the kinetics of activation.

Expression of mRNA that encodes the class C cardiac L-type calcium channel $\alpha 1$ subunit ($\alpha 1_{C1}$) in *Xenopus* oocytes alone is sufficient to direct efficient synthesis of a functional calcium channel that is modulated by dihydropyridines (127). Similarly, mRNAs that encode the alternatively spliced smooth muscle and brain isoforms of the class C $\alpha 1$ subunit direct the synthesis of functional channels in this system (136–138). The $\alpha 2\delta$ subunit substantially increased the amplitude of the calcium current when co-expressed with the cardiac $\alpha 1$ subunit in *Xenopus* oocytes, accelerated its activation and inactivation, and shifted the voltage dependence of inactivation to more negative membrane potentials (127, 139). Co-expression of the cardiac $\alpha 1$ subunit with the β_{1a} subunit in *Xenopus* oocytes increased the peak current, accelerated activation, and shifted the voltage dependence of activation to more negative membrane potentials (139, 140). Similar increases in peak current were observed when the β_{1a} subunit was expressed with the vascular smooth muscle isoform of the cardiac $\alpha 1$ subunit in *Xenopus* oocytes (137) or when the cardiac $\alpha 1$ subunit was expressed with cardiac β_2 subunits (121, 141). Although the β_{1a} subunit did not alter the kinetics of the smooth muscle isoform of the cardiac $\alpha 1$ subunit (137), the cardiac β_2 subunits accelerated activation of the calcium current in oocytes expressing the cardiac $\alpha 1$ subunit and shifted the voltage dependence of activation to more negative membrane potentials (121, 141). β_3 was more effective at increasing current than either β_{2a} or β_{2b}. Thus, the proteins encoded by the different β subunit genes display varied effects on the calcium currents mediated by cardiac $\alpha 1$ subunits.

Co-expression of both $\alpha 2\delta$ and β_{1a} subunits with the cardiac $\alpha 1$ subunit in oocytes resulted in a greater increase in current amplitude, activation at more negative membrane potentials, a steeper voltage dependence of activation and inactivation, and a more rapid current time course compared to expression of the $\alpha 1$ subunit with either of these subunits alone (139, 141). These observations suggest a synergistic interaction between the actions of the $\alpha 2\delta$ and β subunits on the functional properties of the $\alpha 1$ subunit.

The amplitudes of the calcium currents of the $\alpha 1$ subunits of class A, class B N-type, class D L-type, and class E brain calcium channels are greatly increased by co-expression of $\alpha 2\delta$ and β subunits (124, 129, 130, 142–145). The effects of the β subunit are dominant, with less-prominent effects of $\alpha 2\delta$ in most experiments. γ subunits do not have a major effect. In many cases, significant expression of calcium channel function is only observed when auxiliary subunits are co-expressed. However, nuclear injection of expression vectors in *Xenopus* oocytes gives enhanced expression of the $\alpha 1$ subunits alone (129, 138). Under these conditions, the effects of auxiliary subunits on the

function of α1 subunits are observed most clearly, since substantial expression of the α1 subunit alone is achieved for comparison. With the class B, N-type calcium channel, the β_{1b} subunit increased expression of calcium current, increased the rates of activation and inactivation, and shifted the voltage dependence of inactivation toward more negative membrane potentials (129). In addition, co-expression of β_1, β_2, or β_3 subunits with a class A calcium channel α1 subunit causes a progressive increase in the rate of inactivation, which suggests that assembly of α1 subunits with different β subunits during biosynthesis of calcium channels or exchange of β subunits on pre-existing calcium channels may alter their functional properties (143). Like the L-type calcium channels, in which the α1 subunits are functionally autonomous but are modulated by their associated auxiliary subunits, the α1 subunits of neuronal non-L-type calcium channels can function as voltage-gated calcium channels, but they appear to require the presence of associated subunits for efficient functional expression, and expression of different auxiliary subunits alters their functional properties.

Mechanism of Regulation by β Subunits

The importance and multiplicity of the effects of the β subunits on calcium channel function are striking. In general, both the number of high-affinity sites for drug and toxin binding and the amplitude of peak calcium currents are increased, the time course of the calcium current is substantially accelerated, and the voltage dependence of activation and inactivation is altered. What molecular mechanisms can account for these multiple effects? Experiments to date provide some initial clues to possible mechanisms underlying these actions. As noted above, the β_{1a} subunit is a substrate for phosphorylation by numerous protein kinases, and the other β subunits have multiple consensus sites for phosphorylation. Moreover, the β_{1a} subunit is selectively dephosphorylated by phosphoprotein phosphatases (146). Thus, co-expression of different β subunits may induce different regulation by protein phosphorylation/dephosphorylation mechanisms. Since dephosphorylation can influence both the number of active calcium channels and the rate and extent of inactivation of calcium channels (147, 148), the effects of β subunits on these aspects of calcium channel expression and function may be due in part to phosphorylation/dephosphorylation mechanisms. Alternatively, recent experiments show that β subunits also influence the coupling of gating charge movement to channel opening (149). Like other voltage-gated ion channels, depolarization of voltage-gated calcium channels causes a series of voltage-driven transmembrane movements of charged amino acid residues in the α1 subunits that serve as gating charges. Following this movement of gating charges, the calcium channels can open but the probability of opening is much less than 1.0. For the class C α1 subunits expressed in *Xenopus* oocytes, co-expression of β_{2a} increased the efficiency of coupling of

gating charge movement to channel opening by 5-fold. This provocative result suggests that the β subunit can lower the energy barrier for opening the channel pore and can thereby increase peak calcium currents without increasing the number of calcium channels expressed. Since the binding of dihydropyridine drugs to these L-type calcium channels is also influenced by channel state, it is conceivable that the number of high-affinity binding sites observed in expressed channels is also changed by this mechanism without a change in the actual number of calcium channel α1 subunit proteins expressed. This mechanism of action of the β subunits of the calcium channels would be similar to the mechanism of action proposed for the sodium channel β1 subunits that reduce the energy barrier for shift of channel state to a fast gating mode for sodium channels expressed in *Xenopus* oocytes.

A site of interaction of β subunits with α1 subunits has been identified by a novel library-screening method (150). cDNAs that encode segments of the intracellular loop between domains I and II of the α1 subunit were found to express fusion proteins that bind β subunits specifically. Co-expression of the corresponding peptides prevents the effects of β subunits on expression and function of α1 subunits. This segment of the α1 subunit interacts with a conserved motif in the β subunit (151). This interaction domain may mediate the change in energy of activation that increases the efficiency of calcium current expression upon co-expression of β subunits.

INTERACTIONS OF CALCIUM CHANNELS WITH INTRACELLULAR EFFECTOR PROTEINS

Calcium-Release Channels

In skeletal muscle, the L-type voltage-gated calcium channels in the transverse tubule membrane are thought to physically contact the ryanodine-sensitive calcium-release channels in the sarcoplasmic reticulum (SR) membrane. Voltage-driven conformational changes in the voltage-gated calcium channels serve to activate the SR calcium-release channel through protein-protein interactions. Thus, the SR calcium-release channel serves as an effector of the voltage-gated calcium channel in the process of excitation-contraction coupling. Mice with the *muscular dysgenesis* mutation have defective excitation-contraction coupling due to a mutation in the α1 subunit of the skeletal muscle calcium channel (152). Injection of an expression vector encoding the α1 subunit restores excitation-contraction coupling. The site of interaction with the SR calcium-release channel is in large intracellular loop-connecting domains II and III of the α1 subunit (153). Evidently, this loop can transmit a signal from voltage-dependent activation of the transverse tubule calcium channels that can activate the SR calcium-release channel.

Synaptic Membrane Proteins

Neuronal calcium channels in presynaptic nerve terminals initiate the process of neurotransmitter release by allowing the rapid entry of calcium in response to depolarization. The increase in calcium concentration which initiates neurotransmitter release is highly localized, and presynaptic calcium channels and synaptic vesicles are thought to be closely associated in active zones. Recent evidence indicates that this association may be mediated in part through binding of presynaptic calcium channels to synaptic membrane proteins. N-type calcium channels, which are involved in release of neurotransmitters at many synapses, bind to the synaptic plasma protein syntaxin and possibly also to the synaptic vesicle protein synaptotagmin (154–156). Recent studies with fusion proteins representing different segments of the N-type calcium channel α1 subunit show that the site of interaction is in the intracellular loop between homologous domains II and III (157). Thus, the intracellular loop between domains II and III may serve as an effector interaction domain for neurotransmitter release in presynaptic terminals as well as for excitation-contraction coupling in skeletal muscle. Additional interactions for this domain of calcium channel α1 subunits may participate in other cellular signaling processes that are initiated by depolarization or by calcium influx.

STRUCTURE AND FUNCTION OF K^+ CHANNEL SUBUNITS

Principal Subunits of K^+ Channels

Voltage-gated K^+ channels are functionally diverse (158–160). They can be classified into two major groups based on physiological properties: delayed rectifiers which activate after a delay following membrane depolarization and either inactivate slowly or do not inactivate at all, and A-type K^+ channels which are fast-activating and inactivate. The molecular structure of the voltage-gated K^+ channels was first revealed by molecular cloning of the gene encoding the *Shaker* mutation in *Drosophila* (160, 161). A-type K^+ channels and delayed rectifier K^+ channels in *Drosophila* and vertebrates have principal subunits whose polypeptide backbones are 60 to 80 kD and are homologous in structure to a single domain of the α or α1 subunits of Na^+ or Ca^{2+} channels (Figure 2C). They form homotetramers and heterotetramers that are fully functional as voltage-gated ion channels, and they are alternatively spliced adding additional diversity (160, 161). However, several lines of evidence now indicate that, like Na^+ and Ca^{2+} channels, they contain one or more auxiliary subunits as components of their oligomeric structure in situ.

Subunit Composition of Purified Neuronal K^+ Channels

Some brain K^+ channels contain a receptor for dendrotoxins (DTX), a family of neurotoxins isolated from the venom of the black mamba snake,

Dendroaspis polyepsis (162). These basic polypeptide toxins inhibit A-type K^+ channels, resulting in the facilitation of neurotransmitter release and epileptiform activity. The DTX binding site is the receptor for three additional peptide ligands: mast cell degranulating peptide (MCD), β-bungarotoxin (β-BTX), and charybdotoxin (CTX) (162). These toxins have been successfully used as molecular probes to identify and purify components of A-type and delayed rectifier K^+ channels from mammalian brain.

Photo-affinity labeling of potassium channels with β-bungarotoxin reveals peptide components of approximately 75 kDa and 28 kDa in chick brain (163). Purification of DTX-binding proteins from detergent-solubilized mammalian brain membranes revealed a noncovalently associated glycoprotein complex containing polypeptides of 74–80 kD in association with smaller polypeptides of 42 kD, 38 kD, and 35 kD when analyzed by SDS-PAGE (164–166). The 35 kD polypeptide was observed in variable amounts and may be a proteolytic fragment of one of the other polypeptides (164). Binding of [^{125}I]DTX and [^{125}I] MCD to the purified receptor was inhibited by β-BTX, demonstrating that K^+ channels with binding sites for all three ligands were co-purified. The 80 kD DTX-binding protein from rat brain represents a family of pharmacologically and structurally related glycoproteins (164, 167, 168). Neuraminidase treatment reduced its apparent molecular weight to 70 kD, and subsequent treatment with endoglycosidase F further reduced this to 65 kD, indicating that the 80 kD subunit is a sialylated membrane glycoprotein that is exposed to the extracellular surface. In contrast, treatment of the 38 kD polypeptide with neuraminidase or endoglycosidase F had no effect on its mobility on SDS-PAGE, indicating that this component most likely did not contain N-linked sugars (168, 169). The N-terminal amino acid sequence of the 80 kD subunit from bovine brain was virtually identical to that deduced from the cDNA of the mouse/rat homologue of the *Shaker* family of K^+ channels, RCK 5 (MK2/RBK2), a K^+ channel protein from rat brain known to be a DTX-sensitive, delayed rectifier (166, 168). This conclusively demonstrated that the DTX receptor protein is a K^+ channel.

Antibodies against a fusion protein generated from a cDNA that encodes a delayed rectifier K^+ (drk) channel polypeptide from rat brain (170) were used to identify its protein components. Similar to K^+ channels identified via their sensitivity to DTX, this putative delayed rectifier K^+ channel also contained a low molecular weight subunit. Anti-drk antibodies specifically immunoprecipitated a complex of 130 kD and 38 kD polypeptides. Because the antibodies recognized the 130 kD polypeptide exclusively and co-precipitated the 38 kD subunit, it was concluded that these two polypeptides were immunologically distinct proteins in a heterooligomeric complex with a 1:1 stoichiometry. Thus, both delayed rectifier and A-type neuronal K^+ channels may have β subunits (Figure 1C).

Molecular Properties of an Auxiliary Subunit of a K^+ Channel

Recent work by Scott et al. (171) has resulted in the cloning and primary structure determination of the first potassium channel auxiliary subunit. The β subunit of the dendrotoxin-sensitive potassium channel from bovine brain is a protein of 367 amino acids (41 kD) which has no hydrophobic segments and is not glycosylated. Its amino acid sequence contains several consensus sites for protein phosphorylation, and the protein is rapidly phosphorylated by cAMP-dependent protein kinase. Analysis of probable secondary structure reveals four major probable alpha helical segments. In all of these respects, the potassium channel β subunit closely resembles the calcium channel β subunit. However, no extended amino acid sequence homology is detectable, indicating that the evolutionary relationship between the two proteins is distant.

Like the calcium channel β subunits, potassium channel β subunits have important functional effects when co-expressed with α subunits. The time courses of both activation and inactivation are accelerated (172). Moreover, multiple potassium channel β subunits have been identified and they have differential effects on function of the α subunits. Thus, the potassium channel β subunits have a functional analogy with calcium channel β subunits as well as a general structural similarity. It will be of interest to determine whether the mechanisms of action of these two classes of auxiliary subunits are similar.

STRUCTURAL BASIS FOR ION CHANNEL FUNCTION

Purification, molecular cloning, and determination of the primary structures of the principal subunits of sodium, calcium, and potassium channels have provided a molecular template for probing the relationship between their structure and function [reviewed in (2, 173, 174)]. The structure of the principal subunits of each of these channels is based on the same motif (Figure 2; see Figure 4 also): four homologous transmembrane domains which contain six probable transmembrane alpha helices and surround a central ion pore. In potassium channels, each domain is a separate gene product and the functional channel is a homo- or hetero-tetramer. In sodium and calcium channels, four domains are contained within a single functional α or α1 subunit. 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. This section reviews the current status of research on the molecular basis of the three major elements of voltage-gated ion channel function: voltage-dependent activation, ion conductance, and inactivation.

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 that 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 (175). The gating current for sodium channels was 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 even larger gating charge movements. 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 (2, 173, 176). These segments contain repeated motifs of a positively charged residue followed by two hydrophobic residues. For comparison among different channels, these positive charges have been numbered sequentially from the extracellular end of the segment (Figure 3). 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 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 (177). 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 multiple 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.

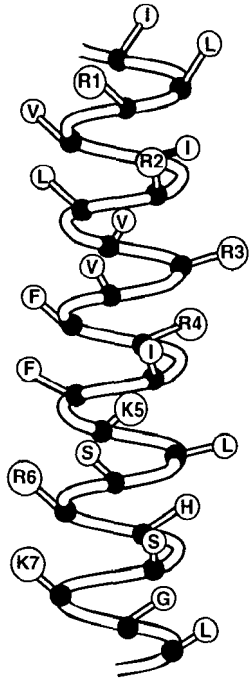


Figure 3 An S4 segment of a K^+ channel. The S4 segment of the Shaker K^+ channel of *Drosophila* is illustrated in a ball-and-stick alpha helical model. Amino acids are illustrated in single-letter code and positively charged amino acids in every third position are numbered from the extracellular end of the helix.

Neutralization of the positively charged residues in the S4 segments of potassium channels also caused reduction in apparent gating charge and shifts of the voltage dependence of activation (178–180). For the *Shaker* potassium channel of *Drosophila,* neutralization of R1 (Figure 1B) causes an unexpectedly large reduction in apparent gating charge while charge reversal has little additional effect (179, 180). Most mutations at this site caused a positive shift in the voltage dependence of gating (179, 180). In contrast, neutralization and charge reversal at R2 and K7 caused changes in apparent gating charge that were closely correlated with the changes in charge caused by the mutations (179, 180), and neutralization of R3 causes a substantial reduction in one component of gating current (181). Analysis of the shifts in voltage dependence caused by mutation of each of the positively charged residues in an S4 helix showed an alternating pattern of voltage shifts for most residues: positive shifts for neutralization in positions 1, 3, 5, and 7; and negative shifts for positions

2 and 4 (182). Only the arginine in position 6 deviates from this pattern. These results are consistent with a model in which all of the gating charges in odd-numbered positions make salt bridges that stabilize the activated state while those in positions 2 and 4 make salt bridges that stabilize the closed state (180, 182). Most mutations that reduce positive charge at R2 caused a negative shift in the voltage dependence of activation while those at K7 caused a positive shift. Overall, the studies of S4 segments in potassium channel gating support the conclusion that the positively charged residues in these segments are indeed gating charges involved in the voltage sensors of the ion channels. The individual residues appear to contribute differentially to the overall apparent gating charge, and their size and chemical properties (other than charge) also have important influences on gating.

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 (183). Similarly, mutation of several hydrophobic residues in the S4 segments of potassium channels also causes dramatic shifts (up to 80 mV) in the voltage dependence of activation (184, 185). In contrast, mutations of several hydrophobic residues in other transmembrane segments did not have major effects on activation (185). 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.

The mechanism by which the S4 segments serve as voltage sensors is not known. The "sliding helix" or "helical screw" models (2, 176) 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 (176). 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. Thus, although the S4 segments are clearly implicated as the voltage sensors of the voltage-gated ion channels, the mechanism through which they initiate activation of the channels remains unknown.

ION CONDUCTANCE

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 in these models 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 (186). These channel blockers therefore serve as molecular markers and specific probes of the pore region of the ion channels. Amino acid residues that 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 (186). 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 (186). These residues have now been identified by site-directed mutagenesis. Noda et al (187) 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 (Figures 1A 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 that were all required for high-affinity tetrodotoxin binding (188). These residues are therefore likely to surround the extracellular opening of the pore and contribute to a receptor site for tetrodotoxin. In addition to the 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

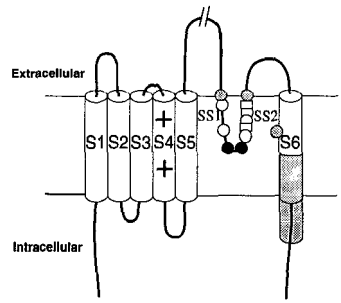


Figure 4 Pore-forming region of the homologous domains of voltage-gated ion channels. The transmembrane folding pattern of a single homologous domain of a voltage-gated ion channel is illustrated as in Figure 1. The short segments SS1 and SS2 and the positions of amino acid residues and segments which have been implicated in pore formation are illustrated. *Open circles,* residues required for ion conductance and selectivity of K^+ channels; *open squares,* residues required for ion conductance and selectivity of Na^+ and Ca^{2+} channels; *shaded circles,* residues required for high affinity binding of pore blockers of K^+ channels; *filled circles,* residues required for both ion conductance and binding of pore blockers of K^+ channels; *shaded bar,* segment of Ca^{2+} channels binding phenylalkylamine pore blockers.

(Figures 1A 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 the amino-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 affinity of the cardiac sodium channel and the converse mutation causes a loss of affinity of 200-fold in the brain or skeletal muscle channel (189–191). 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 (189, 190). 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 (190). Thus, this residue may be

approximately 20% of the way through the electrical field within the pore of the sodium channel.

The outer mouth of the potassium channel has been mapped in a similar way. The polypeptide charybdotoxin is an extracellular blocker of potassium channels which binds in the outer mouth of the pore. Identification of amino acids which contribute to binding of charybdotoxin reveals glutamic acid, aspartic acid, and threonine residues which are required for high-affinity binding (192, 193). The required residues cluster on both sides of the SS1-SS2 region as illustrated in Figure 4, and the residues closest to these short segments are most important for charybdotoxin binding. Conversion of any of these residues to a positively charged amino acid increases the K_d for charybdotoxin binding more than 300-fold, suggesting that positively charged amino acids in the toxin may normally interact with the negatively charged and hydroxylic residues in these positions in the wild-type channel.

Tetraethylammonium ions also block potassium channels from the extracellular side. Analysis of their affinity for block of the same family of potassium channel mutants reveals that residues on both sides of SS1 and SS2 are required, with the amino acid residue in position 449 on the carboxyl terminal side of SS2 being dominant (194). Tyrosine or phenylalanine in this position confers high-affinity block. Carboxyl residues in this position give intermediate affinity, and positively charged residues prevent tetraethylammonium binding completely (194). Phenylalanine residues in this position in all four subunits of a potassium channel can participate in binding tetraethylammonium ion, which suggests that the four phenyl rings coordinate a single tetraethylammonium molecule through cation-π orbital interactions (195, 196). These residues are similar in position in the amino acid sequence to those that are required for tetrodotoxin binding to sodium channels. Thus, it seems likely that tetraethylammonium ions in potassium channels and tetrodotoxin in sodium channels occupy similar receptor regions at the extracellular mouth of the pore when they block the channels.

The Intracellular Mouth of the Pore

Local anesthetics and related antiarrhythmic drugs are thought to bind to a receptor site on the sodium channel that is accessible only from the intracellular side of the membrane and is more accessible when the sodium channel is open (186). Similarly, the phenylalkylamine class of calcium channel antagonists are characterized as intracellular open-channel blockers, and tetraethylammonium and related monoalkyltrimethylammonium derivatives can block potassium channels from the intracellular side of the membrane when the channel is open (186). Both biochemical and molecular biological approaches have been used to probe the peptide segments of the principal subunits of the voltage-gated ion channels that 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_ds for equilibrium binding to purified calcium channels in the range of 30 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 α1 subunit only (197). The site of covalent labeling was located by extensive proteolytic cleavage of the labeled α1 subunit followed by identification of the photolabeled fragments by immunoprecipitation with site-directed antipeptide antibodies (197). All of the covalent label recovered was incorporated into a peptide fragment containing the S6 segment of domain IV of the α1 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 end 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 (197, 198).

Analysis of mutations that alter block of potassium channels from the intracellular side by tetraalkylammonium ions point to both the amino acid residues between SS1 and SS2 and the intracellular end of the S6 segment as components of the intracellular mouth of the pore. Mutation of a critical threonine residue (position 441 in the *Shaker* potassium channel) in the sequence between SS1 and SS2 to the closely related amino acid serine is sufficient to increase the K_d for block of potassium channels by intracellular tetraethylammonium ion 10-fold (199). Tetraethylammonium must traverse only 15% of the membrane electrical field in reaching this binding site from the intracellular solution. Thus, it is likely that this threonine residue forms part of a binding site for tetraethylammonium ions at the intracellular mouth of the potassium channel. Alkyltriethylammonium ions with long carbon chains in the alkyl group also require this threonine residue for high-affinity binding and block. In addition, mutations of a threonine residue near the middle of the S6 segment (position 469 in the *Shaker* channel) to a hydrophobic residue increase the affinity for C8 and C10 alkyltriethylammonium ions (200). A more hydrophobic residue in this position gives higher affinity for the alkyltriethylammonium ions, and the effect is greater for larger alkyl substituents consistent with hydrophobic interactions between residues at this position and the alkyl group of the substituted tetraalkylammonium ion. Thus, in potassium channels as well as calcium channels (197, 198), the S6 segments contribute to formation of the intracellular mouth of the pore and to binding of hydrophobic pore-blocking drugs.

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 (201). These two residues are located approximately 11 Å apart on the same face of the proposed S6 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.

Ion Conductance and Selectivity

Consistent with the idea that the amino acid residues that are required for binding of pore blockers are also required for interaction with permeant ions, changes in these residues have dramatic effects on ion condutance and selectivity. A clear demonstration of the close relationship between the amino acid residues that determine pore blocking properties and those that determine ion conductance and selectivity came from studies of chimeric potassium channels in which the SS1/SS2 region is transferred between channel types that differ in both single-channel conductance and affinity for tetraethylammonium ion at intracellular and extracellular sites (202). Such chimeric channels have the ion conductance, affinity for extracellular tetraethylammonium ion, and affinity for intracellular tetraethylammonium ion specified by the SS1/SS2 region with little effect of the remainder of the channel structure. Small changes in individual amino acids within this region also have dramatic effects on ion selectivity (Figure 4). Changes of threonine to serine or phenylalanine to serine increase the conductance of Rb^+ and NH_4^+ (203). Coordinate changes of leucine to valine and valine to isoleucine in two potentially interacting positions in the deep pore region are responsible for the differences in conductance and binding affinity for intracellular tetraethylammonium ion between chimeric channels which differ in the SS1/SS2 region (204). Deletion of two residues (tyr445, gly446) from the SS2 region of the *Shaker* voltage-gated potassium channel to yield a sequence similar in length to the corresponding region of the distantly related cyclic nucleotide-gated ion channels causes loss of potassium selectivity and increased channel block by divalent cations which are characteristic of the cyclic nucleotide-gated ion channels (205). These results indicate that even small alterations in the amino acid residues in the SS1/SS2 region have crucial effects on ion conductance and selectivity, supporting the conclusion that these residues form part of the lining of the transmembrane pore and interact directly with permeant ions. Moreover, changes in amino acid sequence in this region can also determine ion selectivity properties of different families of potassium channels. It is surprising that so many of the amino acid

residues in this region are hydrophobic and that hydrophobic residues are critical determinants of ion selectivity. It remains to be determined how these residues interact with permeant ions to allow rapid and selective ion conductance.

As for potassium channels, changes in the amino acid residues in the SS1/SS2 region that are important for binding of the pore blocker 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 (188). 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 (186). This property is thought to arise from high-affinity binding of calcium ions to two sites in the ion conductance pathway that 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 sodium channel is sufficient to confer calcium channel-like permeability properties (206). 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 (207, 208). 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

Fast Inactivation of Sodium Channels

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 (175). 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 (175). 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 to 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 (209, 210). In both cases, only one antibody, directed against the short intracellular segment connecting homologous domains III and IV (Figures 1 and 5), 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 at which sodium channels are not inactivated, the antibody bound rapidly and inhibited channel inactivation; at more positive membrane potentials at which the sodium channel is inactivated, antibody binding and action were greatly slowed or prevented. Based on these results, it was proposed that the segment that this antibody recognizes is directly 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 (209, 210) (Figure 5).

A similar model is supported by site-directed mutagenesis experiments (177). 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

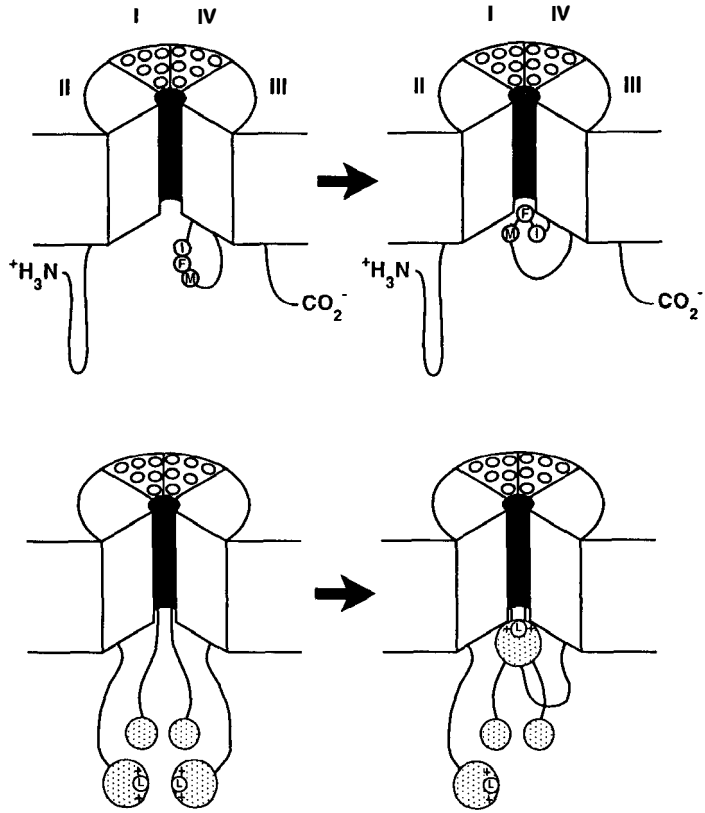


Figure 5 Mechanisms of inactivation of Na^+ and K^+ channels. The hinged-lid mechanism of Na^+ channel inactivation (A) and the ball-and-chain mechanism of K^+ channel inactivation (B) are illustrated. The intracellular loop connecting domains III and IV of the Na^+ channel is depicted as forming a hinged lid. The critical residues leu7 (L) and phe1489 (F) are shown as occluding the intracellular mouth of the pores in the K^+ channel and the Na^+ channel, respectively.

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 II and III 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 (211, 212), 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 (212). In contrast, deletion of the 10-amino acid segment at the amino-terminal end of the loop completely blocks fast sodium channel inactivation (212). Scans of the hydrophobic amino acid residues in this segment of the inactivation gating loop by mutation to the hydrophilic but uncharged residue glutamine show that mutation of the three-residue cluster IFM to glutamine completely blocks fast sodium channel inactivation (213). The single phenylalanine in the center of this cluster at position 1489 in sodium channel II is the critical residue (Figure 5). Conversion of this residue to glutamine is sufficient by itself to almost 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 (214). 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 phe1489 replaced by glutamine (215). 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 that requires the same structure as the inactivation particle itself.

A candidate for the inactivation gate receptor region has been identified by alanine-scanning mutagenesis of the S6 segment in domain IV (216). Mutation of three adjacent hydrophobic residues at the intracellular end of this transmembrane segment to alanine produces Na^+ channels that inactivate incompletely, having greater than 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 in which the fast inactivation process has been disrupted. This site is one helical turn on the intracellular side of the amino acid residues that are required for binding of local anesthetic drugs. This may provide a molecular explanation for the finding that local anesthetic drugs can be trapped in the channel by closure of the inactivation gate (186).

A Hinged-lid Model of Sodium Channel Fast Inactivation

The proposed inactivation gate is a short structured loop of the channel which places the inactivation particle (IFM) close to the mouth of the transmembrane pore. The inactivation gate loop resembles the "hinged lids" of allosteric enzymes that 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 that pivots to place the inactivation particle in a position to bind to the intracellular mouth of the transmembrane pore of the sodium channel (213) (Figure 5). The three-dimensional structures of some hinged lids of allosteric enzymes that 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 Potassium Channels

In contrast to the results obtained with sodium channels, the inactivation particle responsible for rapid inactivation of potassium channels by occlusion of the intracellular mouth of the pore is located at the amino-terminus of the polypeptide (217). Removal of the amino-terminus, by deletion mutagenesis, prevents fast inactivation. Mutations of single positively charged amino acids in that region slow inactivation, and mutation of leu7 to a hydrophilic amino acid prevents fast inactivation almost completely (217). Free peptides with amino acid sequences modeled on the inactivation particle restore fast, voltage-dependent inactivation to mutant potassium channels whose amino-termini have been deleted (218). These results fit the expectations of the "ball-and-chain" model of ion channel inactivation originally proposed for sodium channels by Armstrong and Bezanilla (reviewed in 175). The amino-terminal segment of the potassium channels is envisioned as an inactivation particle tethered on the end of a chain of approximately 200 amino acids (Figure 5). The positively charged and hydrophobic residues in the ball are thought to

interact with an inactivation receptor at the intracellular mouth of the channel through a process of restricted diffusion and binding. This mechanistic model predicts that shortening the chain of amino acids connecting the ball to the rest of the channel should accelerate inactivation, while elongating the chain should slow inactivation. The model also predicts that extracellular permeant ions should oppose inactivation as they diffuse through the pore and compete with the inactivation particle for its binding site. These effects were in fact observed for expressed potassium channels. Fifty residue segments deleted or inserted in the "chain" between the N-terminal inactivation particle and the membrane accelerate or slow inactivation (217). External potassium ions also slow inactivation and speed recovery from inactivation as expected if inactivation cannot occur while the pore is occupied by permeant ions (219). These results support the "ball-and-chain" mechanism as a valid model of potassium channel inactivation.

The "receptor" occupied by the N-terminal inactivation particle as it occludes the mouth of the potassium channel may include amino acid residues in the short intracellular loop connecting transmembrane segments S4 and S5. Mutagenesis of charged residues in this loop to neutral, hydrophilic ones and mutations of hydrophobic residues to alanine markedly reduce fast potassium channel inactivation (220). Mutations in this loop also reduce conductance and alter ion selectivity of the potassium channels (221). Thus, this short intracellular loop may contribute to formation of the intracellular mouth of the pore, along with sequences in the intracellular end of the S6 segment and the SS1/SS2 region as described above, and may serve to form a receptor for the amino-terminal inactivation particle.

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NOTE ADDED IN PROOF

Remarkable progress has been made in the past several years toward definition of the structural elements that are responsible for the basic functions of the voltage-gated ion channels. Beyond the new insights into the structure and function of the individual channels that have been gained, a striking commonality of functional design has emerged that allows a range of channel gating and permeability properties to be derived from subtle variations on a common structural theme. This commonality of function is observed in the common structures for voltage sensing in the S4 segments, the related structures of the pore–forming regions of the channels and the subtle variations in their sequence which dramatically alter ion selectivity, the similar locations for receptor sites for extracellular and intracellular pore-blocking drugs among different channel types, and in the common mechanistic basis for inactivation of sodium and potassium channels, even though the structural basis for inactivation of the two channels is different. Future investigations using the presently available methods of protein chemistry, mutagenesis, functional expression, and molecular modeling should delineate the primary structural basis for the essential functions of the ion channels and for the functional differences between different channel types at ever higher resolution. However, a mechanistic understanding of these processes in terms of protein structure will ultimately require determination of the three-dimensional structure of a member of the ion channel family.