From Ionic Currents to Molecular Mechanisms: The Structure and Function of Voltage-Gated Sodium Channels

Review

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Voltage-gated sodium channels are responsible for the rising phase of the action potential in the membranes of neurons and most electrically excitable cells. Exploration of the molecular properties of voltage-gated ion channels began twenty years ago this past January with report of the discovery of the sodium channel protein by neurotoxin labeling methods (Agnew et al., 1980; Beneski and Catterall, 1980). This review briefly recounts the early biochemical studies of sodium channels, focuses primarily on the emerging view of the molecular mechanisms of sodium channel function and regulation, and gives a perspective for future research on the expanding family of sodium channel proteins. Discoveries of sodium channel diseases, new sodium channel genes, new modes of modulation of sodium channel function, and new forms of sodium channel protein–protein interactions greatly expand the possible roles for sodium channels in neuronal function and regulation.

The Sodium Channel Protein
Discovery and Purification
Sodium currents were first recorded by Hodgkin and Huxley, who used voltage clamp techniques to demonstrate the three key features that have come to characterize the sodium channel: (1) voltage-dependent activation, (2) rapid inactivation, and (3) selective ion conductance (Hodgkin and Huxley, 1952). Detailed analysis of sodium channel function during the 1960s and 1970s using the voltage clamp method applied to invertebrate giant axons and vertebrate myelinated nerve fibers yielded mechanistic models for sodium channel function (Armstrong, 1981; Hille, 1984). These functional studies predicted that sodium channels would be rare membrane proteins, difficult to identify and to isolate from the many other proteins of excitable membranes.

During the 1970s, a new line of research emerged, focussing on development of methods for molecular analysis of sodium channels. Biochemical methods for measurement of ion flux through sodium channels, high affinity binding of neurotoxins to sodium channels, and detergent solubilization and purification of sodium channel proteins labeled by neurotoxins were progressively developed (reviewed in Ritchie and Rogart, 1977; Catterall, 1980). These biochemical approaches led to discovery of the sodium channel protein in 1980. Photoaffinity labeling with a photoreactive derivative of an α-scorpion toxin identified the principal α subunit (260 kDa) and the auxiliary β1 subunit (36 kDa) of brain sodium channels (Beneski and Catterall, 1980). Immediately thereafter, partial purification of tetrodotoxin binding proteins from electric eel electroplax revealed a correlation between

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tetrodotoxin binding activity and a protein of $\sim$270 kDa (Agnew et al., 1980). Subsequent purification studies showed that the sodium channel from mammalian brain is a complex of α (260 kDa), β1 (36 kDa), and β2 (33 kDa) subunits (Hartshorne and Catterall, 1981; Hartshorne et al., 1982), the tetrodotoxin-binding component of the sodium channel purified from eel electroplax is a single protein of 270 kDa (Miller et al., 1983), and the sodium channel from skeletal muscle is a complex of α and β1 subunits (Barchi, 1983). Reconstitution of neurotoxin-activated ion flux through purified sodium channels of known subunit composition from brain and skeletal muscle revealed that they contain a functional pore (Talvenheimo et al., 1982; Kraner et al., 1985), and fusion into planar bilayer membranes demonstrated voltage-dependent activation (Hartshorne et al., 1985; Furman et al., 1986). Together, these studies showed that the purified sodium channel protein contained the essential elements for ion conduction and voltage-dependent gating and opened the way for more detailed molecular analysis of the channel protein.

Primary Structure and Functional Expression
Using both oligonucleotides encoding short segments of the electric eel electroplax sodium channel as well as antibodies directed against the channel protein, Noda et al. (1984) isolated cDNAs encoding the entire polypeptide from expression libraries of electroplax mRNA. Their work gave the initial insight into the primary structure of a voltage-gated ion channel. The deduced amino acid sequence revealed a large protein with four internally homologous domains, each containing multiple potential α-helical transmembrane segments (Figure 1B). The structural motif of the sodium channel homologous domains is the building block of the voltage-gated calcium and potassium channels and a large family of related ion channels, including the cyclic nucleotide-gated channels, calcium-activated potassium channels, and *trp*-like calcium channels. The wealth of information contained in this deduced primary structure revolutionized research on the voltage-gated ion channels.

Although the primary structure of the eel electroplax sodium channel provided the template for molecular analysis, successful functional expression of the sodium channel required cloning cDNAs from rat brain. RNA encoding the α subunit proved sufficient for functional expression of sodium currents in *Xenopus* oocytes (Goldin et al., 1986; Noda et al., 1986a, 1986b), but the β subunits are required for normal kinetics and voltage dependence of gating (Isom et al., 1992, 1995). The β1 and β2 subunits of sodium channels have similar overall structures but are not closely related in amino acid sequence. Both β subunits have a large, glycosylated extracellular domain, a single transmembrane segment, and a small intracellular domain (Figure 1; Isom et al., 1992, 1995).

Application of molecular modeling to the sodium channel led to a remarkably accurate prediction of the two-dimensional folding pattern of the α subunit, even before any experimental analysis of structure and function was available (Guy and Seetharamulu, 1986). This
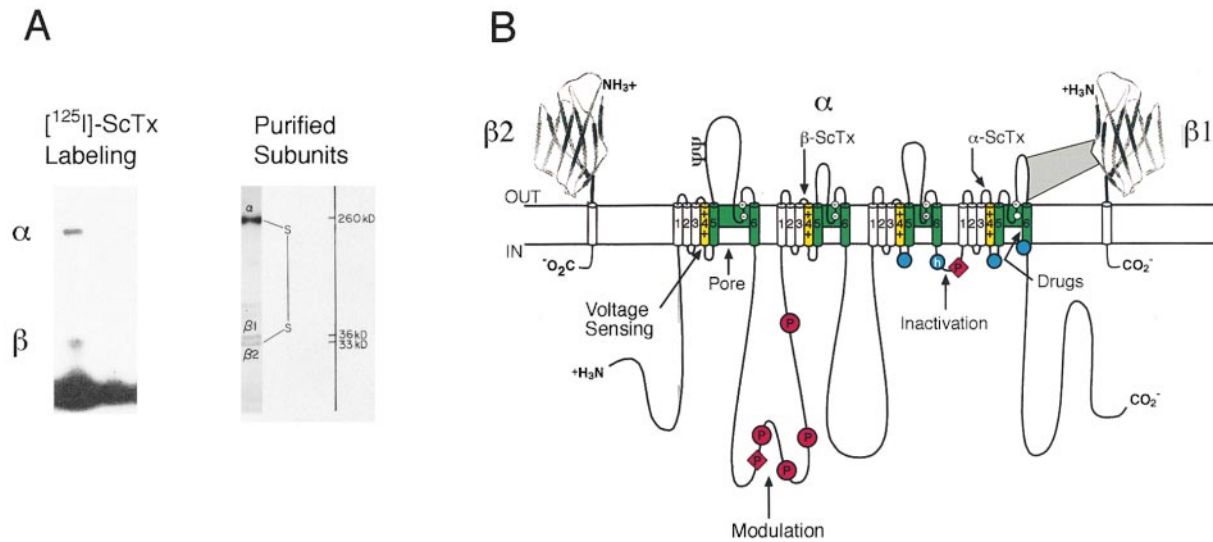
Figure 1. Subunit Structure of the Voltage-Gated Sodium Channels

(A) SDS-polyacrylamide gel electrophoresis patterns illustrating the α and β subunits of the brain sodium channels. Left, α and β1 subunits covalently labeled with [^{125}I]scorpion toxin (Beneski and Catterall, 1980). Lane 1, specific labeling; lane 2, nonspecific labeling. Right, sodium channel purified from rat brain showing the α, β1, and β2 subunits and their molecular weights (Hartshorne et al., 1982). As illustrated, the α and β2 subunits are linked by a disulfide bond.

(B) The primary structures of the subunits of the voltage-gated ion channels are illustrated as transmembrane folding diagrams. Cylinders represent probable α-helical segments. Bold lines represent the polypeptide chains of each subunit with length approximately proportional to the number of amino acid residues in the brain sodium channel subtypes. The extracellular domains of the β1 and β2 subunits are shown as immunoglobulin-like folds. ψ, sites of probable N-linked glycosylation; P in red circles, sites of demonstrated protein phosphorylation by PKA (circles) and PKC (diamonds); green, pore-lining segments; white circles, the outer (EEDD) and inner (DEKA) rings of amino acid residues that form the ion selectivity filter and the tetrodotoxin binding site; yellow, S4 voltage sensors; h in blue circle, inactivation particle in the inactivation gate loop; blue circles, sites implicated in forming the inactivation gate receptor. Sites of binding of α- and β-scorpion toxins and a site of interaction between α and β1 subunits are also shown.

analysis predicted six α-helical transmembrane segments (S1–S6) in each of the four homologous domains (I–IV) and a reentrant loop that dipped into the transmembrane region of the protein between transmembrane segments S5 and S6 and formed the outer pore (Figure 1B). Relatively large extracellular loops were predicted in each homologous domain, connecting either the S5 or S6 transmembrane segments to the membrane-reentrant loop. Even larger intracellular loops were predicted to connect the four homologous domains, and large N-terminal and C-terminal domains were also predicted to be intracellular. Subsequent work on sodium, calcium, and potassium channels is consistent with the general features of this early model.

Comparison of the primary structures of the auxiliary β1 and β2 subunits to those of other proteins revealed a probable structural relationship to the family of proteins that contain immunoglobulin-like folds (Isom et al., 1995). The extracellular domains of the β1 and β2 subunits are predicted to fold in a similar manner as myelin protein p0, whose structure is known (Figure 1B; Shapiro et al., 1996). The immunoglobulin-like fold is a sandwich of two β sheets held together by hydrophobic interactions. To date, the β1 and β2 subunits are unique among ion channel subunits in containing immunoglobulin-like folds.

The Molecular Basis for Sodium Channel Function
The Outer Pore and Selectivity Filter
Just as the high affinity and specificity of neurotoxins led to discovery of the sodium channel protein, the pore blockers tetrodotoxin and saxitoxin also led directly to identification of the outer pore and selectivity filter. Voltage clamp studies provided a model of tetrodotoxin and saxitoxin as plugs of the selectivity filter in the outer pore of sodium channels (Hille, 1984). Mutational analysis identified glutamate 387 in the membrane-reentrant loop in domain I as a crucial residue for tetrodotoxin and saxitoxin binding (Noda et al., 1989), and subsequent studies revealed a pair of important amino acid residues, mostly negatively charged, in analogous positions in all four domains (Figure 1B, small white circles; Terlau et al., 1991). These amino acid residues were postulated to form outer and inner rings that serve as the receptor site for tetrodotoxin and saxitoxin and as the selectivity filter in the outer pore of sodium channels. This view derives strong support from the finding that sodium channels can be made calcium selective by exchanging the amino acid residues in the inner ring from DEKA in domains I–IV to their counterparts EEEE in calcium channels (Heinemann et al., 1992b). Mutations in this postulated ring of four amino acid residues have strong effects on selectivity for organic and inorganic monovalent cations, in agreement with the idea that they form the selectivity filter (e.g., Schlief et al., 1996; Sun et al., 1997).

These conclusions about the structure of the outer pore of sodium channels also derive strong support from work on potassium channels. A corresponding pore loop was identified in potassium channels and shown to control ion conductance and selectivity (Miller, 1991), and
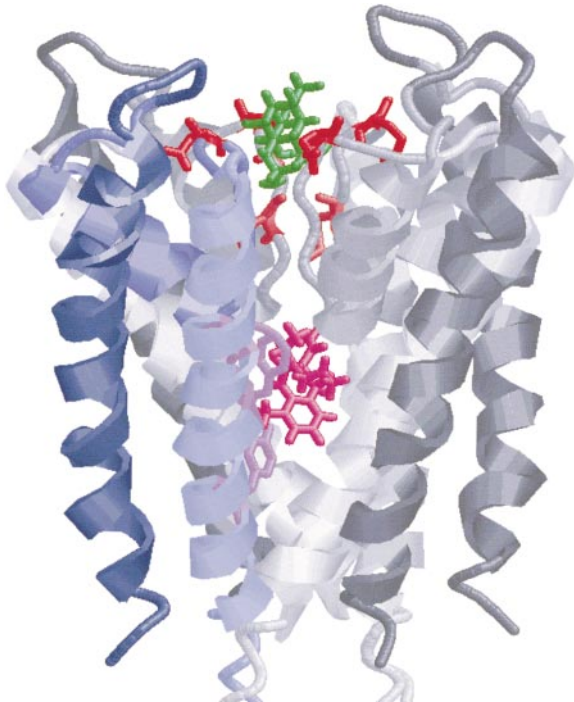
Figure 2. A Model of the Pore of the Sodium Channel

The α-helical fold of the Kcsa potassium channel (Doyle et al., 1998) is used as a model for the sodium channel pore. Domain IV of the sodium channel is in blue while others are in gray. M2 segments (equivalent to S6) are in light gray or light blue. M1 segments (equivalent to S5) are in darker gray or blue. The amino acid residues of the selectivity filter in the outer pore are superimposed on the potassium channel structure: outer ring of acidic residues (EEDD) inner ring of DEKA residues in red. Tetrodotoxin (green) is shown approaching the outer mouth of the pore. Phe-1764 and Tyr-1771, which form part of the local anesthetic receptor site, are illustrated in dark blue in transmembrane segment IVS6, and etidocaine illustrated in purple is shown approaching the local anesthetic receptor site in the cavity in the inner pore.

the three-dimensional structure of a bacterial potassium channel reveals a narrow ion selectivity filter formed by the pore loop from its four identical subunits (Doyle et al., 1998). The two rings of amino acid residues that form the sodium channel selectivity filter are illustrated superimposed on the potassium channel structure in Figure 2 (red). However, in the potassium channel, the backbone carbonyl groups of the peptide bonds of three consecutive amino acid residues form the narrow part of the selectivity filter, in contrast to the suggestion from mutagenesis studies of sodium channels that the carboxyl side chains of glutamate and aspartate residues interact with permeant ions and determine ion selectivity. It will be of great interest to learn how the structure of the outer pore of potassium channels is modified to yield the different ion selectivity of sodium and calcium channels.

All sodium channels studied to date have similar permeation properties and are therefore expected to have similar selectivity filters. However, cardiac sodium channels bind tetrodotoxin with 200-fold lower affinity because of a change of a tyrosine or phenylalanine, which

is located two positions preceding the first pore glutamate in domain I in the brain and skeletal muscle channels, to cysteine in the cardiac sodium channel (Backx et al., 1992; Heinemann et al., 1992a; Satin et al., 1992). Serine in this position in some peripheral nervous system sodium channels causes even larger decreases in tetrodotoxin binding affinity (Sivilotti et al., 1997). Thus, a single amino acid residue in the selectivity filter in domain I determines the affinity of different sodium channel types for the pore blocker tetrodotoxin. Cadmium is a high-affinity blocker of cardiac sodium channels but not of brain or skeletal muscle sodium channels because of its interaction with this cysteine residue (Backx et al., 1992; Satin 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 ~20% of the way through the electrical field within the selectivity filter in the pore of the sodium channel.

Although the pore structure of ion channels is most often considered to be static, substituted cysteine mutagenesis and cross-linking experiments suggest that the pore loops of sodium channels are asymetrically organized and dynamic on the millisecond time scale of sodium channel gating (Benitah et al., 1999). Cysteine residues substituted in analogous pore positions in domains I–IV are at different positions in the electric field. The domain II pore loop is most superficial, domains I and III intermediate, and domain IV most internal, as judged by the voltage dependence of cadmium block of the mutant channels (Chiamvimonvat et al., 1996). Cross-linking of pairs of substituted cysteine residues reveals unexpected flexibility of the pore regions, allowing formation of disulfide bonds between both distant and closely apposed amino acid residues (Pérez-García et al., 1996). These cross-linking reactions are enhanced by activation of sodium channels with repetitive pulses, suggesting that increased motion during gating brings potentially reactive cysteine residues close together to allow disulfide bond formation (Benitah et al., 1999). Movement of the selectivity filter region on the millisecond time scale of channel gating is therefore likely. An additional important question is whether these regions can also move on the microsecond time scale of ion conductance.

The Inner Pore and Local Anesthetic Receptor Site

Voltage clamp studies led to the conclusion that local anesthetics enter from the intracellular side and bind in the inner pore of sodium channels (Hille, 1984), and similar work revealed analogous intracellular block of calcium and potassium channels. As for the outer pore, the first indication that the S6 segments form the inner pore of the voltage-gated ion channels came from locating a pore-blocker receptor site—in this case the phenylalkylamine receptor site of L-type calcium channels (Striessnig et al., 1990). Photoaffinity labeling with these high-affinity, intracellular pore blockers showed that only the IVS6 segment of the calcium channel $\alpha 1$ subunit was labeled. Subsequently, mutagenesis studies of sodium channels revealed the local anesthetic receptor site in an analogous position in the sodium channel

(Ragsdale et al., 1994). High-affinity binding of local anesthetics to the inactivated state of sodium channels requires two critical amino acid residues, Phe-1764 and Tyr-1771 in brain type IIA channels, which are located on the same side of the IVS6 transmembrane segment two α-helical turns apart (Figure 2, Phe-1764 and Tyr-1771 in blue, etidocaine in purple). It is likely that the tertiary amino group of local anesthetics interacts with Phe-1764, which is located more deeply in the pore, and that the aromatic moiety of the local anesthetics interacts with Tyr-1771, which is located nearer to the intracellular end of the pore (Figure 2, blue residues). Subsequent work in several laboratories has shown that sodium channel blocking drugs of diverse structure that are used as antiarrhythmic drugs and as anticonvulsants also interact with the same site as local anesthetics but make additional interactions with other nearby amino acid residues as well.

Substituted tetraethylammonium derivatives, which are intracellular blockers of certain potassium channels, interact with similar amino acid residues in the S6 transmembrane segment (Choi et al., 1993). These amino acid residues are located in an aqueous cavity within the pore of the Kcsa bacterial potassium channel (Doyle et al., 1998), providing additional support for this location of the local anesthetic receptor site in sodium channels.

Voltage-Dependent Activation

The voltage dependence of activation of the sodium channel and other voltage-gated ion channels derives from the outward movement of gating charges in response to changes in the membrane electric field (Hodgkin and Huxley, 1952; Armstrong, 1981). Recent studies indicate that ~12 electronic charges in the sodium channel protein move across the membrane electric field during activation (Hirschberg et al., 1995). The novel features of the primary structure of the sodium channel α subunit led directly to hypotheses for the molecular basis of voltage-dependent gating (Catterall, 1986; Guy and Seetharamulu, 1986). The S4 transmembrane segments contain repeated motifs of a positively charged amino acid residue followed by two hydrophobic residues, potentially creating a cylindrical α helix with a spiral ribbon of positive charge around it (Figure 3, left). The negative internal transmembrane electrical field would exert a strong force on these positive charges arrayed across the plasma membrane, pulling them into the cell in a cocked position. The sliding helix (Catterall, 1986) or helical screw (Guy and Seetharamulu, 1986) models of gating propose that these positively charged amino acid residues are stabilized in the transmembrane environment by forming ion pairs with negatively charged residues in adjacent transmembrane segments (Figure 3, right). Depolarization of the membrane was proposed to release the S4 segments to move outward along a spiral path, initiating a conformational change that opens the pore. Although this model was quite speculative when it was proposed, its major features have now received direct experimental support from work on both sodium and potassium channels.

The first evidence in favor of the S4 segments as voltage sensors came from mutagenesis studies of sodium channels (Stuhmer et al., 1989). Neutralization of the positively charged residues in the S4 segment of domain I was found to reduce the steepness of voltage-dependent gating, as expected for a reduction in gating charge, and to shift the voltage dependence along the voltage axis (Stuhmer et al., 1989). Mutations in the positively charged residues in other S4 segments also have strong effects, especially at the fourth position (Kontis et al., 1997). Thus, this functional test supports the idea that the positive charges in S4 segments are the gating charges.

The proposed outward movement of the S4 segments of sodium channels has been detected directly in cleverly designed mutagenesis and covalent modification experiments. Yang, George, and Horn (Yang and Horn, 1995; Yang et al., 1996) used sodium channel mutants with cysteine residues substituted for positively charged amino acid residues. Modification of these substituted cysteine residues with charged sulfhydryl reagents caused detectable changes in the functional properties of the channel, which could be used to monitor the rate and extent of cysteine reaction. Measurements of reaction rate as a function of membrane potential showed that the substituted cysteine residues became progressively more accessible to reaction with extracellular reagents and less accessible to reaction with intracellular reagents as the membrane was depolarized. Remarkably, these experiments showed that three positively charged amino acid residues in the S4 segment of domain IV become accessible outside the cell during channel gating. This is substantially more transmembrane movement of the S4 segment than predicted in the original sliding helix or helical screw models of gating but is consistent with the more recent measurements of movement of 12 gating charges for the entire sodium channel with four S4 segments. To accommodate this large movement, Yang et al. postulated that the S4 segments move in specialized channels through the protein that have a narrow waist to allow small outward movements to translocate gating charges all the way across the permeability barrier to ions and small reagents (Figure 3, right). In this way, smaller S4 movements could account for the large changes in cysteine accessibility and the large gating charge movements recorded experimentally.

The transmembrane movement of the S4 segments of potassium channels has also been measured by covalent labeling of substituted cysteine residues (Larsson et al., 1996; Yusaf et al., 1996) and by proton transport induced by substitution of histidine residues in the helix (Starace et al., 1997). Histidine residues can protonate and deprotonate at physiological pH. Therefore, the transmembrane movement of the S4 helix could potentially generate a proton current. This proton current has been detected in voltage clamp experiments and has the same voltage-dependent properties as the gating current of potassium channels (Starace et al., 1997).

Experiments on potassium channels also add indirect support for two additional aspects of the sliding helix or helical screw models of gating—formation of ion pairs and rotational movement of S4. Neutralization of the positive charges in S4 by formation of ion pairs is supported by site-directed genetic complementation and rescue experiments (Papazian et al., 1995; Tiwari-Woodruff et al., 1997). Neutralization of single negatively

Figure 3. Voltage-Dependent Activation by Outward Movement of the S4 Voltage Sensors

Left, amino acids in the S4 segments in domain IV of the sodium channel are illustrated in single letter code. Hydrophobic residues are indicated in light blue, and positively charged arginine residues in every third position are highlighted in yellow. Right, a synthesis of the sliding helix or helical screw models of gating (Catterall, 1986; Guy and Seetharamulu, 1986) with the idea that the S4 segments move through narrow channels in each domain of the sodium channel protein (Yang et al., 1996). The S4 segment in a single domain is illustrated as a rigid cylinder within a channel with a narrow, hourglass-shaped waist. Positively charged residues (yellow) are neutralized by interaction with negatively charged residues from the surrounding protein (principally the S2 and S3 segments). Upon depolarization, the segment moves outward and rotates to place the positively charged residues in more outward positions but is still neutralized by interactions with negative residues in the transmembrane part of the protein.

charged amino acid residues in the S2 and S3 transmembrane segments of potassium channels prevents maturation and glycosylation of the potassium channel protein and such mutants are retained in the endoplasmic reticulum and degraded. Paired neutralization of single charged amino acid residues within the S4 segment rescues expression of functional channels. Successful mutation pairs neutralize negatively charged and positively charged residues in similar transmembrane positions in the S4 and either the S2 or S3 segments. These negatively charged amino acid residues are highly conserved among voltage-gated ion channels (Keynes and Elinder, 1999). Thus, the results support formation of a specific set of ion pairs to stabilize the S4 segments in the membrane.

Rotational movement of the S4 segments as they move outward is supported by recent disulfide cross-linking experiments (Sivaprasadarao et al., 1999). Cysteine residues introduced into the S4 segments of homotetrameric potassium channels cause a voltage-dependent block of channel gating in the presence of extracellular cysteine oxidizing agents that catalyze the formation of disulfide bonds. Depolarization enhances the cysteine reaction, suggesting that S4 segments are cross-linked as they emerge from the transmembrane region of the sodium channel during the gating process. Cysteine residues substituted in multiple positions around the proposed α helix can form disulfide bonds, suggesting that the S4 α helices rotate with respect to each other to bring these residues into close proximity during the gating process. Resonance energy transfer experiments between fluorophores covalently attached to specific amino acid residues in different locations in potassium channels also suggest rotational movement of S4 segments with respect to the channel protein during activation gating (Cha et al., 1999b; Glauner et al., 1999). Though indirect, these results are consistent with an outward

rotational movement of the S4 segments, as postulated in the sliding helix or helical screw gating models (Catterall, 1986; Guy and Seetharamulu, 1986).

The outward gating movement of the S4 segments during channel activation is also a target for neurotoxin action. β-scorpion toxins, which enhance sodium channel activation by shifting its voltage dependence to more negative membrane potentials, bind to a receptor site near the extracellular end of the IIS4 transmembrane segment (Cestèle et al., 1998). Toxin binding alone has no effect on activation, but when the channel is activated by depolarization, the bound toxin enhances activation by negatively shifting the voltage dependence. This effect is thought to be mediated by trapping the activated S4 voltage sensor in its outward, activated position by binding to the toxin sitting in its receptor site on the extracellular surface of the channel protein (Cestèle et al., 1998). Voltage-sensor trapping appears to be a widespread mechanism through which channel gating is altered by polypeptide neurotoxins, including the effects of α-scorpion toxins on sodium channel inactivation described below (Rogers et al., 1996).

Inactivation

Sodium channels inactivate within a few milliseconds of opening. Although inactivation is presented here as the last of the basic functional features of sodium channels, genetic diseases that are caused by impairment of sodium channel inactivation attest to its physiological importance (see below), and the sodium channel inactivation gate was the first functional component of an ion channel to be identified experimentally. Based on its sensitivity to proteases perfused inside the squid giant axon, fast inactivation was thought to be mediated by an intracellular gate that binds to the intracellular mouth of the pore and was modeled as a ball tethered to the intracellular surface of the channel by a flexible chain (Armstrong, 1981). Site-directed anti-peptide antibodies

against the short, highly conserved intracellular loop connecting domains III and IV of the sodium channel α subunit (Figure 1) but not antibodies directed to other intracellular domains were found to prevent fast sodium channel inactivation (Vassilev et al., 1988, 1989). Moreover, the accessibility of this site for antibody binding was reduced when the membrane was depolarized to induce inactivation, suggesting that the loop connecting domains III and IV forms an inactivation gate which folds into the channel structure during inactivation (Vassilev et al., 1988, 1989). Cutting the loop between domains III and IV by expression of the sodium channel in two pieces greatly slows inactivation (Stuhmer et al., 1989). Mutagenesis studies of this region revealed a hydrophobic triad of isoleucine, phenylalanine, and methionine (IFM) that is critical for fast inactivation (Figure 1B, blue circle with h; West et al., 1992), and peptides containing this motif can serve as pore blockers and can restore inactivation to sodium channels having a mutated inactivation gate (Eaholtz et al., 1994). These results support a model in which the IFM motif serves as a tethered pore blocker that binds to a receptor in the intracellular mouth of the pore. Inactivation is impaired in proportion to the hydrophilicity of amino acid substitutions for the key phenylalanine residue (F1489), suggesting that it forms a hydrophobic interaction with an inactivation gate receptor during inactivation (Kellenberger et al., 1997b). Voltage-dependent movement of the inactivation gate has been detected by measuring the accessibility of a cysteine residue substituted for F1489 (Kellenberger et al., 1996). This substituted cysteine residue becomes inaccessible to reaction with sulfhydryl reagents as the inactivation gate closes. Glycine and proline residues that flank the IFM motif may serve as molecular hinges to allow closure of the inactivation gate like a hinged lid (Figure 4A; Kellenberger et al., 1997a).

The three-dimensional structure of the central portion of the inactivation gate has been determined by expression as a separate peptide and analysis by multidimensional NMR methods (Rohl et al., 1999). These experiments reveal a rigid α helix flanked on its N-terminal side by two turns, the second of which contains the IFM motif (Figure 4B). The fold of the inactivation gate peptide projects F1489 into the solvent away from the core of the peptide, an unusual position for a hydrophobic residue in a short peptide. In this position, F1489 is poised to serve as a tethered ligand that occludes the pore. The nearby threonine (T1491), which is an important residue for inactivation (Kellenberger et al., 1997b), also is in position to interact with the inactivation gate receptor in the pore. In contrast, the methionine of the IFM motif (M1490) is buried in the core of the peptide, interacting with two tyrosine residues in the α helix. This hydrophobic interaction stabilizes the fold of the peptide and forces F1489 into its exposed position. The structure of the inactivation gate peptide in solution suggests that the rigid a helix serves as a scaffold to present the IFM motif and T1491 to a receptor in the mouth of the pore as the gate closes.

Scanning mutagenesis experiments have revealed multiple amino acid residues that may form the inactivation gate receptor within and near the intracellular mouth of the pore (Figure 1B, blue circles), including hydrophobic residues at the intracellular end of transmembrane
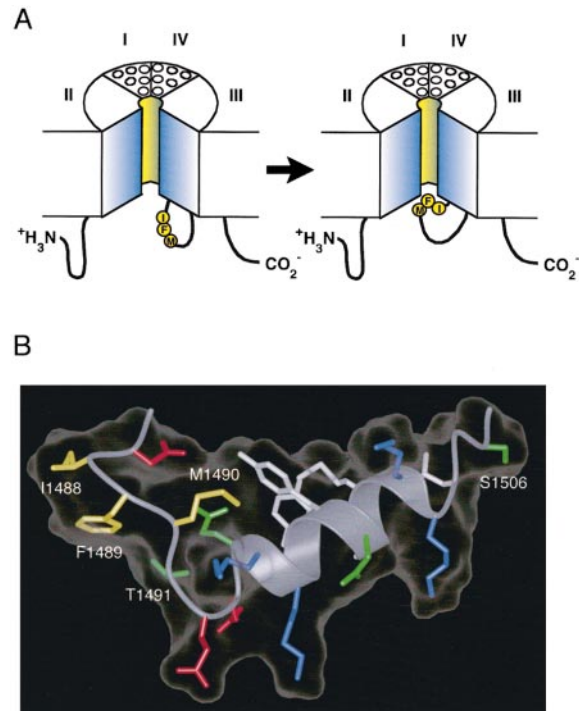

Figure 4. Mechanism of Inactivation of Sodium Channels

(A) The hinged-lid mechanism of Na^+ channel inactivation is illustrated. The intracellular loop connecting domains III and IV of the Na^+ channel is depicted as forming a hinged lid. The critical residue Phe-1489 (F) is shown occluding the intracellular mouth of the pore in the Na^+ channel during the inactivation process.
(B) Three-dimensional structure of the central segment of the inactivation gate as determined by multidimensional NMR (Rohl et al., 1999). Ile-1488, Phe-1489, and Met-1490 (IFM) are illustrated in yellow. Thr-1491, which is important for inactivation, and Ser-1506, which is a site of phosphorylation and modulation by protein kinase C, are also indicated.

segment IVS6 (McPhee et al., 1995) and amino acid residues in intracellular loops IIIS4–S5 (Smith and Goldin, 1997) and IVS4–S5 (Lerche et al., 1997; Filatov et al., 1998; McPhee et al., 1998; Tang et al., 1998). Mutations of residues in each of these positions impair inactivation by destabilizing the inactivated state, as expected for disruption of the inactivation gate receptor. In addition, mutations in intracellular loop IVS4–S5 impair closed channel block by IFM-containing peptides, consistent with function as the inactivation gate receptor (McPhee et al., 1998), and paired insertions of charged residues in the IIIS4–S5 loop and in the IFM motif indicate that these peptide segments interact during inactivation (Smith and Goldin, 1997). Evidently, multiple peptide segments form a complex inactivation gate receptor into which the inactivation gate closes to occlude the inner pore.

Coupling of Activation to Inactivation
Sodium channel inactivation derives most or all of its voltage dependence from coupling to the activation process driven by transmembrane movements of the S4 voltage sensors (Armstrong, 1981). Increasingly strong evidence implicates the S4 segment in domain IV in this process. Mutations of charged amino acid residues at

the extracellular end of the IVS4 segment have strong and selective effects on inactivation (Chen et al., 1996). α-scorpion toxins and sea anemone toxins uncouple activation from inactivation by binding to a receptor site at the extracellular end of the IVS4 segment and preventing its normal gating movement (Rogers et al., 1996; Sheets et al., 1999), evidently trapping it in a position that is permissive for activation but not for fast inactivation. The IIIS4 and IVS4 segments, detected by covalently incorporated fluorescent probes, are specifically immobilized in the outward position by fast inactivation, arguing that their movement is coupled to the inactivation process (Cha et al., 1999a). Together, these results provide strong evidence that outward movement of the S4 segment in domain IV is the signal to initiate fast inactivation of the sodium channel by closure of the intracellular inactivation gate. The molecular mechanism for coupling of this movement of IVS4 to gate closure is an interesting subject for further investigation.

The β Subunits

The β1 and β2 subunits of the sodium channel appear to have dual functions—modulation of channel gating and cell–cell interaction. Expression of the α subunits of brain or skeletal muscle sodium channels alone in *Xenopus* oocytes yields sodium currents that activate and inactivate more slowly than expected from recordings of dissociated neurons or myocytes. Coexpression of the β1 and β2 subunits accelerates channel gating to normal rates (Isom et al., 1992, 1995; Bennett et al., 1993; Schreibmayer et al., 1994). The effect of the β1 subunit is mediated by the immunoglobulin-like fold in the extracellular domain (Chen and Cannon, 1995; Makita et al., 1996; McCormick et al., 1998), which is sufficient to modulate channel gating when attached to an unrelated transmembrane segment or to a glycophospholipid anchor without the transmembrane and intracellular domains (McCormick et al., 1999). Analysis of two different sets of channel chimeras points to the loop on the extracellular side of transmembrane segment IVS6 as one important point of interaction of the β1 subunit (Figure 1B; Makita et al., 1996; Qu et al., 1999). Interactions with this extracellular loop serve to modulate channel activation and coupling to fast inactivation via an unknown mechanism.

A surprising finding from determination of the primary structures of the β1 and β2 subunits (Isom et al., 1992, 1995) was their close structural relationship to the large family of cell adhesion molecules that mediate cell–cell interactions in the nervous system and other tissues (Vaughn and Bjorkman, 1996). No other auxiliary subunits of ion channels have related structures. In cell adhesion molecules, immunoglobulin-like folds bind extracellular proteins and thereby influence neuronal migration, axonal extension, and interactions with the substrate and other cells. The structural similarity of the β subunits to cell adhesion molecules suggests that they perform similar functions, and direct experimental support for this idea has come from recent experiments on interaction of the β subunits with extracellular proteins. Sodium channels and the β2 subunit bind to the extracellular matrix proteins tenascin-C and tenascin-R (Srinivasan et al., 1998). Transfected cells expressing sodium

channel subunits are repelled by surfaces coated with tenascin R, as though interaction with this extracellular protein is a repellent signal to migrate away from the interacting surface (Xiao et al., 1999). These interactions may guide the formation of specialized areas of high-sodium channel density such as nodes of Ranvier and axon initial segments and may stabilize the high density of sodium channels in these locations.

Neuromodulation

Early voltage clamp studies of sodium channels in axons and in skeletal muscle fibers did not reveal regulation of the sodium current by cellular second messenger pathways, perhaps because of dialysis of the cytoplasm in these experimental preparations. However, biochemical studies of sodium channels in brain synaptosomes showed that they are rapidly phosphorylated by cAMP-dependent protein kinase, which reduces their ion conductance activity (Costa and Catterall, 1984a). Sodium channels are phosphorylated by PKA in intact neurons (Rossie and Catterall, 1987) on four sites in the intracellular loop connecting domains I and II (Figure 1B; Murphy et al., 1993). Consistent with the biochemical studies, phosphorylation of these sites reduces peak sodium currents in brain neurons and in cells expressing cloned sodium channels without substantially altering the voltage dependence of activation and inactivation (Li et al., 1992; Smith and Goldin, 1996). Moreover, action potential generation and peak sodium currents are reduced by dopamine acting on D1-like receptors and subsequent activation of the cAMP signaling pathway (Calabresi et al., 1987; Surmeier and Kitai, 1993; Cantrell et al., 1997). The primary effects are due to phosphorylation of a single serine residue (Cantrell et al., 1997; Smith and Goldin, 1997), and anchoring of PKA near the sodium channel by binding to an A kinase anchoring protein (AKAP-15) is required for regulation (Cantrell et al., 1999b). Although the significance of sodium channel regulation in vivo is not yet well defined, there is clear evidence for involvement in dopamine regulation of the firing properties and input-output relationships of striatonigral neurons (Surmeier and Kitai, 1993). In addition, sodium currents in nucleus accumbens neurons are persistently reduced after treatment with cocaine, a drug which increases dopaminergic neurotransmission by blocking dopamine reuptake (Zhang et al., 1998). This effect on sodium channels correlates with the symptoms of anergia, anhedonia, and depression that accompany cocaine treatment (Zhang et al., 1998). These results provide the first in vivo evidence for potential behavioral consequences of neuromodulation of sodium channels.

Sodium channels are also rapidly phosphorylated by protein kinase C (Costa and Catterall, 1984b), and activation of protein kinase C by diacylglycerols or by acetylcholine acting through muscarinic acetylcholine receptors slows sodium channel inactivation and reduces peak sodium currents (Sigel and Baur, 1988; Lotan et al., 1990; Numann et al., 1991; Cantrell et al., 1996). The slowing of sodium channel inactivation results from phosphorylation of a site in the inactivation gate (Figure 1B; West et al., 1991), while the reduction in peak sodium current requires phosphorylation of sites in the intracellular loop between domains I and II, as observed for

Table 1. The Sodium Channel Protein Family

Names[a]	Sequence Identity (Percent)	Human Chromosome	Primary Site of Expression	Genbank Access
Type 1	87	2	CNS	X03638
Type II/IIA	100	2	CNS	X03639
Type III	87	2	CNS	Y00766
μ1, Skm1	82	17	Skeletal muscle	M26643
h1, Skm2	77	3	Heart	M27902
Type VI, SCN8a	88	12	CNS	L39018, F049240
PN-1, hNe, Nas	76		PNS	U79568
PN-3, SNS	78		PNS	X92148, U53833

[a] The names given to the cloned sodium channel α subunits by the original authors are listed. There is no significance to the sequence of listing of the names. Sequence identity is for rat sodium channels compared to type II, as specified by the authors of the original cloning papers. Three additional sodium channel-related proteins expressed in the PNS have been cloned and sequenced but not yet functionally expressed (Genbank Accession Numbers M91556, M96578, Y09164, L36179, and AF059030).

PKA modulation (Figure 1B; Cantrell et al., unpublished data). Both phosphorylation of the sodium channel by PKC and steady membrane depolarization enhance the effects of concomitant PKA phosphorylation (Li et al., 1993; Cantrell et al., 1999a), resulting in a complex, convergent regulation of sodium channel function by these three distinct signaling pathways. It is likely that these neuromodulatory processes serve to tune the overall excitability and input-output relationships of central neurons in response to changes in their incoming synaptic activity.

Sodium channels also are modulated by tyrosine phosphorylation. Activation of receptor tyrosine kinases in PC12 cells causes a negative shift in the voltage dependence of sodium channel inactivation (Hilborn et al., 1998). Moreover, sodium channels bind the transmembrane receptor tyrosine phosphatase RPTPβ through both extracellular and intracellular interactions with α and β1 subunits (Ratcliffe et al., 1999). The extracellular carbonic anhydrase domain and the intracellular phosphatase domains of RPTPβ both bind to sodium channels. Sodium channels in transfected cells are phosphorylated on tyrosine residues, and association with the intracellular domains of RPTPβ causes a positive shift of the voltage dependence of channel inactivation, effectively reversing the effects of tyrosine phosphorylation (Ratcliffe et al., 1999). These results demonstrate a functional sodium channel signaling complex containing RPTPβ, which is well designed to regulate sodium channel function in response to cell–cell and cell–matrix interactions. Such regulation of sodium channel function may act in concert with the interactions of β subunits with extracellular matrix proteins and may be important in the formation or function of specialized domains containing high concentrations of sodium channels.

The Sodium Channel Protein Family

Early electrophysiological studies of sodium channels did not reveal very much diversity in their functional properties (reviewed in Hille, 1984), so a large family of sodium channel genes was not expected. However, as illustrated in Table 1, at least eight genes encoding sodium channel α subunits are now known whose encoded proteins have been expressed and functionally analyzed and three more close relatives are known that have not yet been functionally expressed (reviewed in Goldin,

1999). Sodium channel types I, II, III, and VI are very similar to each other in amino acid sequence and are highly expressed in brain and spinal cord. Sodium channel type μ1 is primarily expressed in skeletal muscle, and sodium channel type h1 is primarily expressed in adult heart and developing or denervated skeletal muscle. The newly discovered sodium channel types are expressed primarily in the peripheral nervous system. PN-1 is broadly expressed, while PN-3 is primarily expressed in dorsal root ganglion neurons. The restricted expression of PN-3 sodium channels in dorsal root ganglion neurons is surprising considering the broad expression of other sodium channel types throughout the central nervous system. These results suggest that other novel sodium channel genes may be specifically expressed in individual classes of neurons with specialized functions.

Sodium Channelopathies

Perhaps the most unexpected spin-off of research on the molecular properties of sodium channels has been the discovery that they are the targets for mutations that cause multiple inherited diseases of hyperexcitability in humans. The first sodium channelopathies to be discovered were the familial periodic paralysis syndromes hyperkalemic periodic paralysis (Ptácek et al., 1991; Rojas et al., 1991) and paramyotonia congenita (McClatchey et al., 1992; Ptácek et al., 1992), which are caused by numerous dominant mutations in the skeletal muscle sodium channel. These mutations cause hyperactive sodium channels by impairing the inactivation mechanism, altering the voltage dependence of activation, or slowing the coupling of activation to inactivation. Mutations have been found in the inactivation gate itself, in regions thought to serve as the inactivation gate receptor, and in the S4 segment in domain IV, which is thought to couple activation to inactivation (Figure 5). This gain-of-function of the mutant sodium channels leads to a dominant phenotype, because only a few percent of noninactivating sodium current is sufficient to cause repetitive firing and steady depolarization that lead to periodic paralysis.

Mutations which impair inactivation of cardiac sodium channels also lead to a disease of hyperexcitability— inherited long QT syndrome type 3. In this disease, a
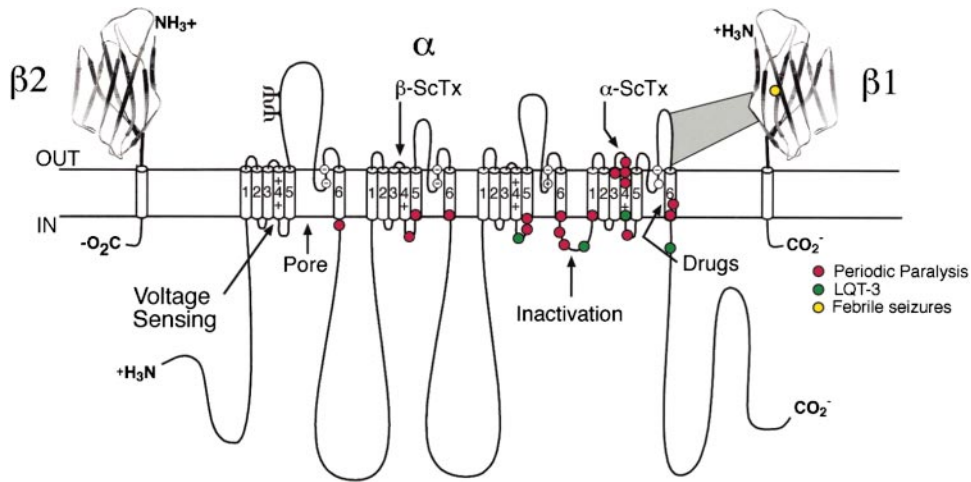
Figure 5. Sodium Channel Mutations that Cause Diseases of Hyperexcitability

The sodium channel subunits are illustrated as transmembrane folding diagrams. Sites of mutations that cause human diseases are illustrated. Red, mutations in the skeletal muscle sodium channel that cause periodic paralyses; green, mutations in the cardiac sodium channel that cause long QT syndrome type 3; yellow, mutation in the $\beta 1$ subunits that causes familial febrile seizures.

small percentage of noninactivating sodium current persists during the plateau of the cardiac action potential and prolongs it. In the electrocardiogram, this prolongs the interval between the QRS complex and the T wave, hence the name long QT syndrome. Patients with long QT syndrome are at greatly increased risk of life-threatening ventricular arrhythmias. As for the periodic paralyses, mutations in long QT syndrome type 3 alter amino acid residues in the inactivation gate and in the inactivation gate receptor region and thereby impair channel inactivation (Figure 5; Bennett et al., 1995; Wang et al., 1995).

Although no mutations in α subunits of neuronal sodium channels have been found to cause inherited disease to date, a mutation in the $\beta 1$ subunit causes inherited febrile seizures by altering the function of brain sodium channels (Wallace et al., 1998). The single mutation described to date alters one of the cysteine residues that stabilize the immunoglobulin-like fold in the extracellular domain of the $\beta 1$ subunit. As the immunoglobulin-like fold in the extracellular domain of the $\beta 1$ subunit is responsible for its modulation of sodium channel gating (McCormick et al., 1998; McCormick et al., 1999), it is not surprising that this mutation diminishes the functional effect of the $\beta 1$ subunit on brain sodium channels expressed in *Xenopus* oocytes (Wallace et al., 1998). These results show that modulation of sodium channel gating by the $\beta 1$ subunit is essential for normal sodium channel function in vivo. One can anticipate that subtle mutations in the α subunits of brain sodium channels may cause inherited epilepsies as well, and recent evidence suggests that a second form of inherited febrile seizures maps to a location on human chromosome 2 near a cluster of α subunit genes (Baulac et al., 1999).

Although the inherited diseases caused by sodium channel mutations afflict only a small number of individuals, they provide novel insights into disease mechanisms that may be relevant for complex idiopathic diseases of hyperexcitability that are widespread, such as

cardiac arrhythmia and epilepsy. The first example of such mutations in a nonfamilial disease is idiopathic ventricular fibrillation (Chen et al., 1998). Analysis of patient samples for genetic lesions by DNA sequencing detected three mutations. One of these mutations accelerated the recovery from sodium channel inactivation. Because mutations that impair sodium channel inactivation are dominant, gain-of-function mutations, it is likely that more examples of sodium channel mutations will be discovered that predispose individuals to diseases of hyperexcitability when combined with environmental stress or with other mutations that alter physiological homeostasis.

The sodium channelopathies also give novel insights into the structure and function of sodium channels in vivo. Mutations that cause the periodic paralyses in skeletal muscle and long QT syndrome in heart cluster in regions of the channel that form the inactivation gate, the inactivation gate receptor, and the voltage sensors that initiate activation and its coupling to inactivation (Figure 5). Thus, regions of the channel protein that have been identified in vitro using site-directed antibodies and site-directed mutagenesis are found to be critical determinants of channel gating in vivo as well. Remarkably, mutations that cause only a few percent of noninactivating sodium current are sufficient to lead to these diseases of hyperexcitability (Cannon, 1996). Evidently, fast and complete sodium channel inactivation is absolutely required for normal physiological function of excitable cells in vivo.

Perspectives

Looking to the future, one can see several new trends that will grow in importance in research on sodium channels. Work over the past decade has been dominated by structure-function analysis. Work on this aspect of sodium channels will undoubtedly continue, but with increasing emphasis on determination of the three-dimensional structure of functional components of the channel

protein, like the inactivation gate. While determination of the complete three-dimensional structure of a sodium channel is likely to remain a challenge for many years because of the size, complexity and difficulty of expression of large amounts of pure protein, one can anticipate that rapid advances in protein crystallography will eventually overcome these formidable technical obstacles and provide an atomic-level template for more definitive analysis of sodium channel structure and function.

Recent results indicating that sodium channels may serve as targets for cell–cell and cell–matrix interactions suggest new avenues of research in neuronal development and cell biology. Generally, the proteins that receive, generate, and transmit electrical signals in neurons have been thought to be largely independent of those that mediate cell–cell and cell–matrix interaction. Direct interaction of sodium channels with tenascin and receptor protein tyrosine phosphatase β, which are implicated in neuronal development, axonal pathfinding, and synapse formation, suggests that sodium channels and other molecules involved in neuronal signal generation and propagation may also participate directly in the protein–protein interactions that determine neuronal development and cell connectivity. This new role for sodium channels may allow the excitability of the cell to be regulated by its immediate extracellular environment.

The recent discoveries of new sodium channel subtypes open unexpected possibilities for both neurophysiology and pharmacology. Novel sodium channel subtypes may mediate persistent sodium currents or back-propagating action potentials in dendrites, aspects of neuronal electrogenesis that have not yet been assigned to specific sodium channel types. The novel sodium channels expressed specifically in dorsal root ganglion neurons have been rapidly established as molecular targets for development of drugs for treatment of chronic neuropathic pain. With the precedent of the novel sodium channels expressed specifically in sensory neurons, a more determined search for other unexpected sodium channel types in specialized neurons seems likely to be productive.

Sodium channel dysfunction in inherited diseases of hyperexcitability is now well established as noted above. However, only a single example of an idiopathic disease of hyperexcitability caused by sodium channel mutations has been described to date. One may anticipate that other dominant mutations in sodium channels may predispose individuals to multiple forms of epilepsy and perhaps even to neurodegenerative diseases in which hyperexcitability may endanger sensitive neurons and increase their vulnerability to other potentially damaging influences.

The functional role in vivo of neuromodulation of sodium channel function by neurotransmitters acting through intracellular signal transduction pathways, including the cAMP and protein kinase C pathways, is also an important direction for new advances. Regulation of sodium channel function is an ideal mechanism for cellular plasticity; that is, change in the responsiveness of the entire neuronal cell to its inputs and resulting change in its input-output relationships. This form of plasticity is not well studied compared to synaptic plasticity but has the potential to play a crucial role in more global aspects of behavior such as emotion, mood, and affect. Looking ahead, one can only hope that as much progress is made on these newer aspects of the role of sodium channels in neuronal function as the past twenty years have yielded on channel structure and function.

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