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Structure, Function and Expression of Voltage-Dependent Sodium Channels

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Abstract

Voltage-dependent sodium channels control the transient inward current responsible for the action potential in most excitable cells. Members of this multigene family have been cloned, sequenced, and functionally expressed from various tissues and species, and common features of their structure have clearly emerged. Site-directed mutagenesis coupled with in vitro expression has provided additional insight into the relationship between structure and function. Subtle differences between sodium channel isoforms are also important, and aspects of the regulation of sodium channel gene expression and the modulation of channel funtion are becoming topics of increasing importance. Finally, sodium channel mutations have been directly linked to human disease, yielding insight into both disease pathophysiology and normal channel function. After a brief discussion of previous work, this review will focus on recent advances in each of these areas.

Index Entries: Voltage-dependent ion channel; sodium channel diseases; site-directed mutagenesis.

Introduction

Since the original electrophysiologic description of sodium currents during an action potential (Hodgkin and Huxley, 1952), enormous advances have been made in our understanding of the structure and function of voltage-dependent sodium channels. The sodium channel protein has been purified, reconstituted, sequenced, functionally expressed, and selectively mutated. With these advances, the understanding of the molecular workings of this complex protein now extends to three dimensional domain-based structural models. The details of the structural differences that distinguish sodium channel isoforms and account for their specialized function, as well as the factors that govern isoform expression, are beginning to emerge.

In this review the focus will be on recent studies of sodium channel structure, function, and expression. We will concentrate on the interplay of electrophysiology, biochemistry, immunology, and molecular biology which has led to our current concepts of this molecule. A vast literature covering earlier electrophysiologic and biochemical studies of the sodium channel will be only briefly summarized; there are available a number of excellent reviews (Armstrong, 1981; Hille, 1984; Horn, 1984; Yamamoto, 1985; Catterall, 1986, 1988; Barchi, 1988; Cohen and Barchi, 1992b).

Early Studies of Structure and Function

Sodium Currents and Channel Kinetics

The fundamental role of sodium currents in the production of action potentials was first defined by Hodgkin and Huxley (Hodgkin and Huxley, 1952). They demonstrated that the rapid upstroke of the action potential is the consequence of a voltage-dependent increase in membrane sodium conductance that shifts the membrane potential from a point near the potassium equilibrium potential (V_K ~ –90 mV) through zero toward the sodium equilibrium potential (V_{Na} ~ +55 mV). This conductance increase is transient, lasting only milliseconds, and then spontaneously inactivates, allowing the membrane to repolarize as potassium permeability once again predominates. In many excitable membranes, repolarization is enhanced by the delayed activation of a second channel specific for potassium ions.

The application of the voltage clamp technique to a variety of different excitable cells corroborated and extended the initial kinetic observations made in the squid giant axon. Voltage clamp currents increase and then decrease in a continuous fashion following a rapid depolarization, leading to the early notion that sodium channels

might act as aqueous pores through the membrane, opening progressively as a function of time following membrane depolarization. The development of patch clamping, with its capability to resolve current flow through single ion channels, revolutionized this view (Sigworth and Neher, 1980; Horn, 1984) by demonstrating that depolarization causes sodium channels to undergo a sudden conversion from the closed state to a unique open state with a characteristic conductance of about 20 pS (Fig. 1). After being open for variable lengths of time, sodium channels close to inactivated states that persist until the membrane is repolarized. The lag observed between depolarization and channel opening reflects kinetic delays imposed by passage through a number of nonconducting closed states.

Single channel recordings indicate that channels remain in the open state only briefly relative to the overall duration of the macroscopic current. At most potentials, the time course of inactivation observed in voltage-clamp currents is a result of the variability in latency to first opening of the channels rather than variation in the duration of channel opening (Aldrich, 1986) and the latency to first opening is a characteristic that differs among different sodium channel isoforms.

The typical sodium channel conductance of ~20 pS corresponds to an ion transport rate of more than 10^7 ions/s/channel, within an order of magnitude of the diffusion-controlled rate of ion movement in free solution (Sigworth and Neher, 1980; Meves and Nagy, 1989). This conductance can be compared with rates of 10^4 ions/s for small antibiotic ion carriers such as valinomycin and 5×10^5/s for turnover numbers of some of the more rapid enzyme reactions, such as carbonic anhydrase. Ion transport mediated by the sodium channel is among the most rapid of protein-mediated processes, consistent with the view that ion movement takes place by diffusion through an aqueous pore rather than by a carrier or pump mechanism.

Sodium channels are highly selective, characteristically permitting preferential influx of Na^+ over K^+ or Rb^+ in a ratio of 1:0.14:0.03 (Hille, 1972). Relative conductance measurements with a variety of small monovalent molecules led Hille to model the selectivity filter of the channel pore as a 3.1 Å × 5.1 Å rectangular orifice lined by oxygen atoms that function as hydrogen bond acceptors (Hille, 1971). Ion movement is blocked by protonation of a site with a pK_a value of ~5.2 (Hille, 1968) suggesting that a carboxyl group may be present in the pore. Interactions of partially hydrated metal cations with a site in this region are postulated to account for ion selectivity.

Protein conformational changes that are driven by the effects of the membrane potential on charged or dipolar protein segments should produce a measurable transmembrane current whether or not ion movement occurs. The kinetics of sodium channel activation suggest that the equivalent of six positive charges move from the intracellular to the extracellular side of the membrane for each sodium channel that opens (Hodgkin and Huxley, 1952). This charge movement, or gating current, was first verified experimentally in the squid giant axon (Armstrong and Bezanilla, 1973, 1974); it provides objective evidence of the changes in the protein structure that accompany activation. Ion movement through the channel can be blocked by toxins without affecting gating currents, but persistent membrane depolarization that inactivates sodium channels eliminates gating charge movement with the same time and voltage dependence. Such observations suggest that elements of the channel protein involved in voltage-dependent activation are separate from those that form the pore.

Inactivation, although dependent on the prior conformational changes in the channel that lead to activation, does not represent a simple reversal of the steps leading to channel opening. Consistent with this notion, the inactivation process can be altered independently of activation. For example, exposure of the cytoplasmic surface of the squid giant axon to pronase, alkaline protease b, or trypsin, eliminates rapid channel inactivation while leaving activation intact (Rojas and Armstrong, 1971; Armstrong et al., 1973; Rojas and Rudy, 1976). Trypsin and alkaline protease b cleave on the carboxyl side of lysine and arginine residues, and intracellular application of chemical reagents that specifically modify argi-

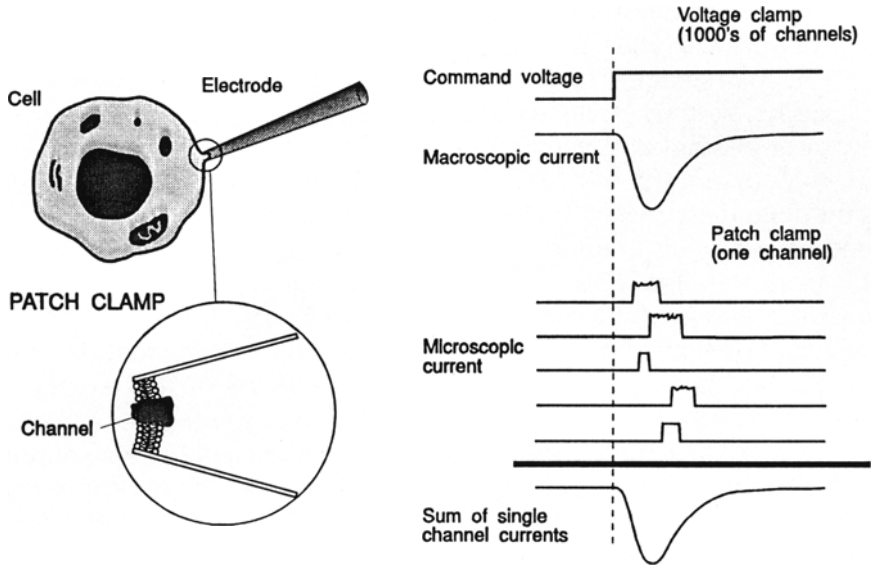

Fig. 1. Voltage-clamp vs patch clamp analysis of sodium currents. When a large area of membrane containing many sodium channels is rapidly depolarized under voltage clamp conditions, an inward sodium current develops, peaks, and smoothly inactivates (**A**). With the patch clamp (**C**), where current through single ion channels can be resolved, individual sodium channels are seen to open rapidly to a characteristic conductance and then abruptly close to an inactivated state (**B**). The smooth macroscopic currents are the statistical summation of stochastic single channel events.

nine residues (glyoxal, phenylglyoxal, and 2,3-butanedione) also irreversibly removes fast inactivation (Eaton et al., 1978). Internal perfusion of the axon with *N*-bromoacetamide and tyrosine-specific reagents (*N*-acetylimidazole, tetranitromethane, and iodide plus lactoperoxidase) also leads to a loss of fast inactivation (Brodwick and Eaton, 1978), but reagents that modify other amino acids have no effect (Oxford et al., 1978). These observations suggest that an intracellular peptide segment containing lysine, arginine, and tyrosine is involved in fast sodium channel inactivation. A slight voltage-dependence of the actions of pronase and *N*-bromoacetamide raises the possibility that some susceptible residues are less accessible to the cytoplasm when the sodium channel is in an inactivated conformation (Salgado et al., 1985).

Channel Pharmacology

A remarkable number of toxins bind to the sodium channel with high specificity and affinity. Binding sites for these toxins have been grouped into six major classes on the basis of electrophysiologic and biochemical measurements (Table 1) (Catterall, 1980). The small, polar, heterocyclic guanidines tetrodotoxin (TTX) and saxitoxin (STX) and the small polypeptide toxin from *Conus geographicus*, μ-conotoxin, bind at Site 1, near the outer entrance to the channel, and block ion flow either by physically occluding the channel or by inducing a conformational change in the protein (Cruz et al., 1985). The binding of both toxins is blocked by protonation of a group with a pK_a value of ~5.4 (Henderson et al., 1973, 1974; Balerna et al., 1975; Reed and Raftery, 1976; Barchi and Weigele, 1979) or by treatment of excitable membranes with carboxyl-modifying reagents (Shrager and Profera, 1973; Baker and Rubinson, 1975, 1976; Reed and Raftery, 1976) suggesting the presence of a carboxyl group in this receptor site. This carboxyl group is different from that responsible for ion selectivity since derivatized channels that no longer bind toxin continue to gate current with normal ion selectivity, although with reduced maximal conductance.

Lipid-soluble alkaloid toxins, including batrachotoxin, veratridine, aconitine, and gray-

Table 1
Drug and Toxin Receptor Sites on the Excitable Membrane Sodium Channel

Binding site	Drug/toxin	Electrophysiologic effect
1	Tetrodotoxin Saxitoxin μ-Conotoxin (GIIIa and GIIIb)	Block ionic conductance by either occluding channel or causing conformational change
2	Veratridine Batrachotoxin Aconitine Grayanotoxin	Shifts the voltage dependence of activation to more negative potentials causing persistent activation
3	α-Scorpion toxins Sea anemone toxins	Shifts the voltage dependence of inactivation to more positive potentials thereby inhibiting inactivation; works synergistically with Site 2 toxins to cause persistent channel activation
4	β-Scorpion toxins	Shifts the voltage dependence of activation to more negative potentials, causing enhanced activation
5	Brevetoxins Ciguatoxins	Shifts the voltage dependence of activation to more negative potentials and block inactivation, causing repetitive firing of nerve cells
6	Goniopora toxin Conus striatus toxin	Inhibit channel inactivation
Binding sites not characterized	Local anesthetics, antiarrhythmics, and anticonvulsants	Frequency- and voltage-dependent inhibition of ionic conductance

anotoxin, bind to Site 2 (Garber and Miller, 1987; Brown, 1988; Behrens et al., 1989). These toxins shift the voltage dependence of channel activation in a hyperpolarizing direction and prevent channel inactivation, resulting in persistent channel activation even at normal membrane potentials.

The α-scorpion and sea anemonae polypeptide toxins, such as anthopleurin-A (AP-A or Ax 1), bind externally to Site 3. These toxins interact allosterically with the binding of toxins at Site 2, slowing or locking inactivation with the net effect of maintaining channels in the active state (Meves et al., 1985; Warashina et al., 1988). α-Scorpion toxin binding is voltage dependent, suggesting that the binding site undergoes a conformational change with depolarization that results in decreased affinity for the toxin.

The β-scorpion toxins, such as *Tityus* γ toxin, bind to a unique extracellular site on the channel (Site 4) where they alter the voltage-dependence of channel activation (Kirsch et al., 1989). In contrast to the binding of toxins to Site 3, β-scorpion toxin binding does not allosterically affect the binding of toxins at Site 2.

Sites 5 and 6 are defined largely on the basis of the actions of toxins that do not fit into the

preceding four classes. Brevetoxin binds externally to Site 5, augmenting the effects of toxins that bind at Site 2 and yet not competing with Site 3 polypeptide toxin binding (Atchison et al., 1986; Poli et al., 1986). The coral toxins from *Goniopora* bind to Site 6 and slow channel inactivation without competing for binding at Site 3 (Gonoi et al., 1986).

Type I antiarrhythmic and local anesthetic agents interact with receptor site(s) from either the aqueous or the lipid phase of the bilayer. Both classes of drugs exhibit "use-dependent" effects, inhibiting sodium currents more rapidly and with greater affinity when membranes are repetitively stimulated (Hille, 1977; Woosley, 1991). This effect is attributed to the voltage-dependent conformational changes associated with channel activation that create or make accessible higher affinity binding site(s) for these drugs when the channel is in the open state. Use-dependence has been rationalized in terms of the "modulated receptor" and "guarded gate" hypotheses of drug binding, which postulate that channel activation allows drug molecules to enter the aqueous pore of the channel and gain better access to specific binding sites from which dissociation is subsequently prevented by the closing of the inactivation gate (Hondeghem and Katzung, 1987). Consistent with this hypothesis is the finding that lidocaine block occurs owing to binding to the inactivated channel (Chahine et al., 1993). A similar situation may apply to the action of the lipid-soluble toxins aconitine and batrachotoxin, which act at sites apparently unrelated to antiarrhythmic/anesthetic agents. Use-dependence for these two toxin groups, which bind at separate noninteracting sites, implies that activation and inactivation involve widespread conformational changes in sodium channel structure. Events at either surface of the channel protein can both affect and be influenced by processes that may be distant in terms of the tertiary structure of the channel.

Biochemistry of the Sodium Channel

All sodium channels that have been isolated and biochemically characterized include a large glycoprotein α-subunit of 240–280 kDa. One or two smaller β-subunits of 30–40 kDa are associated with the α-subunit in some species. The single β-subunit of the rat muscle channel and the β_1-subunit of rat brain are noncovalently associated with the α-subunit, whereas the β_2-subunit found in rat brain is linked through disulfide bonds to the α-subunit (Barchi, 1988; Catterall, 1986, 1988).

Some aspects of the general topology of α-, β_1, and β_2-subunits in the membrane can be inferred from biochemical experiments. Since both α and β channel subunits are heavily glycosylated, both must be at least partially exposed on the extracellular side of the membrane (Elmer et al., 1985; Messner and Catterall, 1985; Roberts and Barchi, 1987; Thornhill and Levinson, 1987). Eel (Costa and Catterall, 1984a,b), rat brain (Costa et al., 1982), rat heart (Cohen and Levitt, 1993), and rat skeletal muscle (Yang and Barchi, 1990) α-subunits are phosphorylated by cAMP-dependent protein kinase both in vivo and in vitro, implying that this subunit penetrates the membrane into the intracellular environment. In the rat brain channel, both the α- and β_1-subunits are covalently labeled by photoaffinity derivatives of scorpion toxins, which are known to bind to the external surface of the channel (Beneski and Catterall, 1980; Sharkey et al., 1984; Jover et al., 1988). The preferential extraction of β_1- and β_2-subunits into hydrophobic detergent phases and their labeling by hydrophobic photoaffinity probes suggests that they contain substantial hydrophobic domains (Reber and Catterall, 1987).

The recently cloned cDNA for a β-subunit from rat brain encodes a protein with a leader sequence and a single transmembrane segment suggesting that at least a portion of the β-subunit extends into the cytoplasm (Isom et al., 1992). cDNAs encoding an identical beta subunit have also been cloned from heart and skeletal muscle, suggesting that the same beta subunit may be associated with the sodium channels expressed in each tissue (Bennett et al., 1993; Yang et al., 1993).

Sodium channels are extensively modified posttranslationally by phosphorylation, sulfation, and fatty acylation in addition to glycosylation. Twenty to thirty percent of the molecular weight of the α- or β-subunits in the eel, rat brain, and

rat skeletal muscle channels is contributed by complex carbohydrate chains containing significant amounts of sialic acid and *N*-acetyl hexosamines (Elmer et al., 1985; Messner and Catterall, 1985; Roberts and Barchi, 1987; Thornhill and Levinson, 1987). Mammalian cardiac sodium channels have lesser amounts of complex carbohydrate (Gordon et al., 1988).

The high degree of glycosylation and the resultant negative charge contributed by sialic acid has led several groups to examine the possible role of these carbohydrate groups in sodium channel function. Inhibition of *N*-linked glycosylation with tunicamycin prevents the insertion of normal amounts of mature sodium channels into the surface membrane of both nerve and muscle cells (Bar-Sagi and Prives, 1983; Waechter et al., 1983; Schmidt et al., 1985; Sherman et al., 1985; Schmidt and Catterall, 1986, 1987). Neurons cultured in the presence of tunicamycin synthesize sodium channel α-subunits in normal amounts but the channel protein is rapidly degraded before being acylated, sulfated, assembled with β_2-subunits, or released from the endoplasmic reticulum. When core glycosylation is allowed to proceed but the subsequent processing and sialylation is inhibited with castanospermine or swainsonine, sodium channel protein is synthesized, otherwise posttranslationally modified, assembled with β-subunits, and inserted into the surface membrane. These sodium channels bind STX and TTX normally, but functional studies were not conducted. Analogous findings were obtained with squid giant axon sodium channels (Gilly et al., 1990). Core glycosylation is required for the normal synthesis, processing, and insertion of sodium channels into the surface membrane, but it is not yet established that channels with only core carbohydrate function normally.

Neuraminidase cleavage of terminal sialic acid groups had no effect on STX and α-scorpion toxin binding but produced channels in which three subconductance states were observed (5, 8, and 14 pS) in addition to the normal one (18 pS) (Scheuer et al., 1988; Recio-Pinto et al., 1990). This treatment also produced a large depolarizing shift in the average potential required for channel activation, implying that sialic acid groups may play a role both in determining the magnitude of the electric field affecting sodium channel activation and in stabilizing the normal conducting conformation of the ionic pore. Without the negatively charged sialic acid residues, the channel may oscillate among a number of closely related conformations, each exhibiting a different mean conductance value.

Posttranslational processing of α-subunits includes thioesterification at cysteine residues by palmitate and the addition of sulfate groups to oligosaccharides (Schmidt and Catterall, 1987). Palmitate incorporation, like core glycasylation, is inhibited by tunicamycin whereas sulfation, like sialylation, is inhibited by castanospermine. Based on these observations, a model of posttranslational processing has been proposed in which core glycosylation occurs in the rough endoplasmic reticulum, allowing proper folding of the sodium channel α-subunit and possibly providing the necessary signal for transport to the Golgi. Although other secondary modifications occur in the Golgi (palmitylation, terminal glycosylation, and sulfation), this structural remodeling is not essential for subunit assembly and transport of functional sodium channels to the cell surface.

Channel Phosphorylation

The rat brain sodium channel alpha subunit is readily phosphorylated by cAMP-dependent protein kinase (pKA). Activation of endogenous pKA in vivo results in phosphorylation of the same residues as are phosphorylated in vitro, suggesting that this covalent modification may play a physiological role (Rossie et al., 1987; Rossie and Catterall, 1989). Supporting the possible importance of phosphorylation, neurotoxin-stimulated $^{22}Na^+$ influx into synaptosomes is reduced in the presence of activators of pKA (Seelig and Kendig, 1982) and whole-cell voltage clamp studies of cultured neurons exposed to pKA activators show that approx 20% of sodium channels inactivate at more negative membrane potentials (Coombs et al., 1988). Peak Na^+ currents are reduced 40% in after pKA phosphorylation in membrane patches from rat brain neurons or from CHO cells expressing the type

IIA channel (Li et al., 1992). When the same rat brain sodium channel (IIA) is expressed in *Xenopus* ooctyes, however, activation of pKA has the opposite effect, increasing the Na^+ current amplitude without affecting this voltage-dependence of activation or inactivation (Smith and Goldin, 1992). Steady-state inactivation of cardiac sodium channels is shifted to more negative membrane potentials in the presence of β-adrenergic agonists, activated G_3, or other agents that increase intracellular cAMP (Schubert et al., 1989). In addition, treatments that increase the intracellular cAMP concentration downregulate the surface expression of sodium channels in cardiac myocytes, although this may involve transcriptional control rather than direct phosphorylation of sodium channels (Taouis et al., 1991b). Finally, cAMP activated cell surface receptors appear to shift the voltage-dependence of cardiac channel activation to more negative potentials (Sorbera and Morad, 1991). In the aggregate these findings clearly suggest that both pKA and cAMP are involved in mediating channel activation, inactivation, and downregulation.

In the rat brain sodium channel, the specific sites of residues phosphorylated by pKA have been identified. These four sites are located within the cytoplasmic loop joining domains 1 and 2 (Rossie and Catterall, 1987, 1989). The rat skeletal muscle alpha subunit is also phosphorylated by pKA, but only at a level of 1 phosphate per channel molecule (Yang and Barchi, 1990).

Sodium channel subunits are also phosphorylated by protein kinase C (pKC) (Costa and Catterall, 1984a; West et al., 1992). Several groups have studied the modulation of sodium channel activity by activators of pKC and here again the results have differed. In both cultured neurons and rat brain IIa, sodium channels were heterologously expressed in CHO cells. Peak sodium currents were decreased by up to 80% by activators of pKC and channel inactivation was markedly slowed. The effects on peak current were attributed to either a decrease in the number of active sodium channels or to a decrease in the probability of single channel opening. The same effects were seen for ensemble currents of excised patches. At the single channel level, the slowing of channel inactivation proved to be the result of both an increased duration of single channel opening and an increased probability of single channel reopening during prolonged depolarizations (Numann et al., 1991). In contrast, others have reported that pKC activation reduced Na^+ currents in *Xenopus* oocytes expressing the same rat brain IIA channel by shifting the activation vs voltage curve to the right (Dascal and Lotan, 1991; Schreibmayer et al., 1991). They did not find any effect on channel inactivation. However, the rat brain IIA channel exhibits abnormally slow inactivation under normal conditions when expressed in oocytes, and this may have obscured any effect of pKC.

The rat brain sodium channel contains a concensus pKC phosphorylation site at ser^{1506}. This residue is located in the highly-conserved cytoplasmic loop joining repeated domains III and IV that has been implicated in channel inactivation. The effects of pKC activation on channel current and inactivation kinetics are both blocked by mutation of ser^{1506} to alanine (West et al., 1991).

Sodium Channel Primary Structure

Alpha Subunit

The complete primary sequence of the α-subunit of sodium channels from various tissues and species has been deduced from molecular cloning experiments and represents a major advance in the structural characterization of sodium channel proteins. cDNAs comprising the coding regions range in size from 6.8 to 9.5 kb and are consistent with mRNA transcripts in the 8.5 to 9.5 kb range. Open reading frames of 1820 to 2072 amino acids correspond to core protein molecular weights of 208 to 228 kDa (Table 2). Sequences for sodium channels from eel (Noda et al., 1984), *Drosophila* (Salkoff et al., 1987), rat and human brain (Auld et al., 1985, 1988; Noda et al., 1986a; Kayano et al., 1988; Ahmed et al., 1992), rat and human skeletal muscle (George et al., 1992b;

Table 2
Cloned Mammalian Sodium Channels

Channel	cDNA, bp	mRNA, kb	Protein length, AA	Predicted core protein, mol wt
Eel electroplax	7,230	8.0	1,820	208,321
Rat brain				
I	8,147	9–9.5	2,009	228,758
II	8,343	9–9.5	2,005	227,840
III	6,822	9.0	1,951	221,375
Rat skeletal muscle	6,957	8.5	1,840	208,847
Rat heart	7,076	8.5	2,018	227,339
Human skeletal muscle	7,823	8.5–9.0	1,836	208,107
Human heart	8,491	9.0	2,016	227,159
Human heart and uterus	7,185	7.8	1,682	193,472
Human brain	8,266	Not reported	2,005	~228,000

Trimmer et al., 1989; Kallen et al., 1990), rat and human cardiac muscle (Rogart et al., 1989; Gellens et al., 1992); rat glial (Gautron et al., 1992), and squid axon (Sato and Matsumoto, 1992a) are now available.

All known sodium channel primary sequences contain four large repeats (231–327 amino acids) that exhibit strong internal homology at the amino acid level. About half of the residues are either identical or conservatively substituted among these domains in each channel, suggesting that these regions of internal homology arose by duplication of a primitive gene element early in evolution. The corresponding repeat domains of sodium channels from different tissues and species are more highly related (80–90%) than are the different domains within a single sodium channel (45–60%), implying that functional distinctions between the different repeat domains evolved prior to species divergence (Trimmer et al., 1989; Kallen et al., 1990; George et al., 1992b). Hydrophobicity analysis of the sequence in each repeat domain identifies six regions capable of forming membrane spanning helices. These are located at the same locations within the sequence of each domain and are designated S1 through S6 beginning at the amino terminal end of each domain. Two of these hydrophobic segments contain no charged residues (S5 and S6), three have several charged residues and are weakly amphipathic (S1, S2, and S3), and one segment (S4) of variable hydrophobicity is unique in containing a positively charged residue at every third position with nonpolar amino acids in the intervening two positions. When the S4 segment is folded into the optimal predicted conformation, the resultant amphipathic α-helix has a spiral band of positive charge along an otherwise neutral surface (Fig. 2). The repeating tripeptide motif (R/K-X-X) occurs four times in D1/S4, five times in D2/S4 and D3/S4, and eight times in D4/S4. The 22 positive charges in the S4 segments are invariant in all sodium channels comprising the major family that have been sequenced and most of the hydrophobic residues in these segments are highly conserved as well.

Inter- and intraspecies sequence homology is generally highest within the repeat domains, whereas interdomain (ID) regions linking the domains exhibit lower homology and contain large segments of amino acids that are present in one species but not in another. This is most evident in the region between D1 and D2 (ID 1–2) where the brain and heart sequences contain a large insertion (~200 amino acids) when compared to the same region of the skeletal muscle

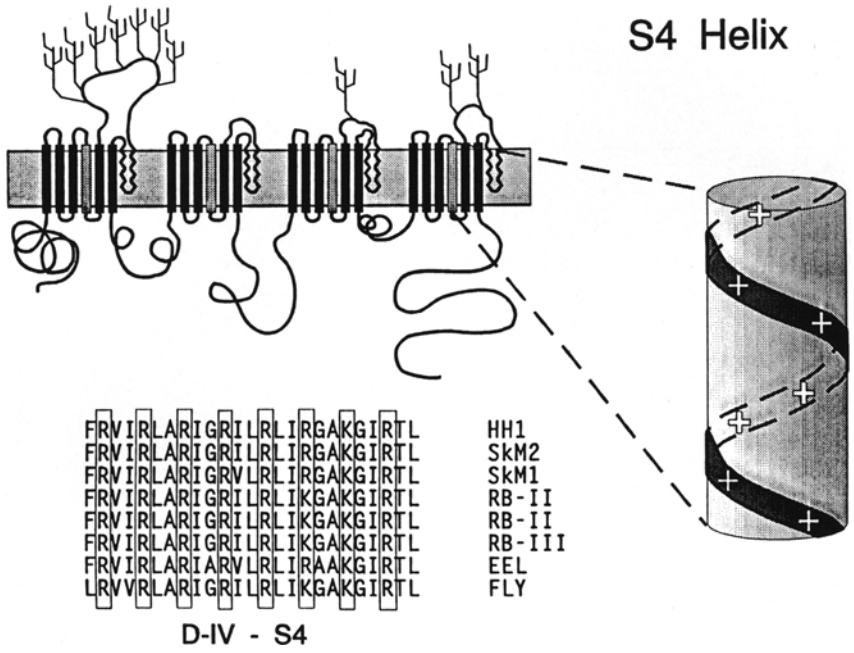

Fig. 2. Proposed structure of S4 helix. The sodium channel alpha subunit contains four regions or repeat domains that exhibit high internal sequence homology. Each contains at least six putative transmembrane helices designated S1–S6. The S4 segment of each domain in all cloned sodium channels contains a highly conserved motif of a positively charged residue at every third position separated by two nonpolar amino acids. When organized as an α-helix, a spiral band of positive charges around an otherwise neutral surface is obtained. These positive charges are thought to pair with fixed negatively charged residues in other helices in each domain. The movement of the positive residues in the membrane electric field is thought to give rise to gating current. The lower panel in this figure demonstrates the marked conservation of both positive and neutral residues in the S4 helices of domain 4 in a variety of sodium channels.

or eel electroplax sequences (Trimmer et al., 1989; George et al., 1992b).

The ID 3–4 region is striking in its almost complete sequence conservation; this represents an exception to the pronounced variability seen in the other interdomain regions, an observation that is consistent with the postulated role for ID 3–4 in channel inactivation. This 53 amino acid segment, by far the shortest of the three ID regions, contains an unusually large number of lysines and arginines, providing multiple targets for agents such as pronase, trypsin, glyoxal, phenylglyoxal, and 2,3-butanedione, which remove inactivation. The two vicinal tyrosines may be the sites at which chymotrypsin and phenol-directed agents act to alter channel inactivation. ID 3–4 has a proline-rich stretch, that may favor pivoting of this short charged segment on the cytoplasmic surface of the channel protein. These observations, as well as site-specific mutations and functional experiments with potassium channels that will be discussed below, have led a number of investigators to propose that ID 3–4 plays a "swinging door" or "ball and chain" role in inactivation gating by blocking the inner mouth of the activated channel (Noda et al., 1986b).

Homologous S4 helices with the same repeating positive charge motif are found in the putative membrane spanning domains of other voltage-sensing proteins, including the α-subunit of voltage-dependent calcium channels (Tanabe et al., 1987) and all of the voltage-dependent potassium channels related to the *Drosophila* potassium channel (e.g., Shaker, Shal, Shab, and

so forth) (Baumann et al., 1987; Kamb et al., 1987, 1988; Papazian et al., 1987; Pongs et al., 1988; Schwarz et al., 1988). Several investigators have independently proposed that this sequence serves as the voltage sensor for these channels and that gating current is the result of movement of these positively charged amino acids within the membrane electric field.

Recently the sequences for two proteins closely resembling these sodium channels have been obtained from glia and heart (George et al., 1992a; Gautron et al., 1992). Although these putative channels retain the same internal domain organization of the sodium channels discussed above, their sequence is more divergent, especially in the S4 segments, suggesting that they may be members of a different but related sodium channel family. These channels have not yet been functionally expressed.

Although most of the segments joining the proposed transmembrane helices in each domain are rather short and variable in amino acid sequence, the loop between helices S5 and S6 in each domain bears particular comment. First, this region is much longer than the other joining segments. In D1, the S5–S6 loop contains large insertions or deletions near its amino terminal end that differentiate various channel isoforms. This loop also contains the sites for glycosylation of the channel. In spite of this variability, the carboxy terminal end of the S5–S6 loop contains two adjacent regions, designated SS1 and SS2, that are highly conserved at the amino acid level among all sodium channels. Homologous regions are also found between the fifth and sixth putative helices in the various potassium channels. Evidence from mutagenesis experiments that will be discussed below support a role for these SS1–SS2 regions in the formation of the lining of the channel pore.

The sodium channel α subunit undergoes extensive posttranslational modifications. All sodium channels that have been fully sequenced have between 15 (rat brain III) and 20 (SkM2) potential *N*-linked glycosylation sites, at least 75% of which are predicted to be extracellular in current models. The majority of these sites are grouped in the first and third domains, and are found nearly exclusively in the loops joining the proposed S5 and S6 helices. Of the *N*-glycosylation sites, five are conserved in all sodium channels, supporting the hypothesis that carbohydrate plays an essential role in channel structure, localization, and/or function.

In terms of potential sites for phosphorylation by cAMP dependent protein kinase, none are universally conserved among channels, although there is a predominance either in the amino terminus, ID 1–2 region, or the carboxy terminus. The rat brain sodium channel has been shown to be a substrate for phosphorylation by protein kinase C (Costa and Catterall, 1984ab; Numann et al., 1991), but phosphorylation by tyrosine kinase has not been reported.

Beta Subunits

Glycosylated and deglycosylated rat brain sodium channel β_1-subunits have apparent molecular masses of 23 and 36 kDa on SDS-PAGE (Messner and Catterall, 1985). Because of technical difficulties in carrying out amino acid sequence studies, the analysis of the β-subunits of the rat brain and skeletal muscle has relied on molecular biological methods. The β_1-subunit of the rat brain sodium channel has been sequenced from cDNA clones and encodes a 218 amino acid protein with a molecular mass ~23 kDa (Isom et al., 1992). Analysis of the sequence leads to a model in which a single transmembrane domain (aa 142–163) is between the extracellular N-terminus and the intracellular C-terminus. This is consistent with the presence of a signal peptide. Four of six potential N-linked glycosylation sites are extracellular in this structural model, and assuming ~3 kDa of carbohydrate is attached to each glycosylation site (~12 kDa total/β_1-subunit), the correspondence of predicted molecular weight with that obtained from biochemical studies is excellent. The proposed β_1-subunit structure is consistent with preferential partitioning into hydrophobic detergent phases and labeling by hydrophobic photoaffinity probes (Reber and Catterall, 1987).

Antibodies against the rat brain beta 1 subunit recognize proteins in the 36–46 kDa range in peripheral nerve, heart, and skeletal muscle as well

(Sutkowski and Catterall, 1990). These differences in apparent molecular weight could represent differences in posttranslational modification of a common protein or the expression of different but structurally related proteins. Recent beta subunit sequences obtained from rat heart and skeletal muscle and human skeletal muscle suggest that the former explanation may hold.

Using PCR methods based on rat brain beta 1 sequence, Bennett et al. (1993) obtained cDNAs encoding a beta subunit from rat heart. This cDNA proved to be identical at the nucleotide level with the brain protein. Yang et al. (1993) have used similar techniques to clone an identical sequence from rat skeletal muscle. cDNAs from human brain and skeletal muscle encode a closely related beta protein that exhibits 96% identity with the rat brain beta 1 and has extensive homology in the 3'-untranslated region as well (McClatchey et al., 1993; Makita et al., 1993). A single gene encodes the $beta_1$ subunit that is expressed in human skeletal muscle, heart, and brain (Makita et al., 1993). Finally, as will be discussed below, coexpression of the beta 1 subunit from different tissues will correct the abnormally slow inactivation of either the rat brain II or the skeletal muscle SkM1 sodium channels in *Xenopus* oocytes.

One sodium channel beta subunit gene appears to be expressed in a variety of different tissues and has the capacity to interact with various different alpha subunit isoforms. Other as yet unidentified beta proteins may be expressed under different physiological conditions or different stages of development. An intriguing possibility is that the properties of a particular alpha isoform might be modified for a given set of conditions by the specific beta subunit with which it associates.

Sodium Channel Tertiary Structure

Structural Models

One major goal of studies of voltage-gated sodium channels is to understand how channel function relates to channel structure. Although electrophysiologic studies have provided considerable insight into the processes of channel gating and ion selectivity, they are not sufficient to describe the molecular mechanisms by which channels operate. Accomplishing this goal will require detailed knowledge of protein structure and of how that structure is affected by membrane potential. Whereas X-ray diffraction of proteins is the most rigorous approach to protein structure currently available, neither crystals of sodium channel protein suitable for diffraction nor sufficient amounts of protein to attempt to make crystals have been obtained.

An alternate approach to this problem is to use the known primary amino acid sequences to model the sodium channel tertiary structure. Although our ability to accurately predict tertiary structure of even moderately sized soluble proteins is still very limited, investigators have embarked on efforts to exploit this method by generating predictions of sodium channel structure that can be tested experimentally.

Analytical methods that predict local secondary structure for short segments of the primary sequence were used to generate the channel models to be discussed below. With the accrual of sequence information for additional sodium channel subtypes as well as for other related voltage-gated ion channels, these models have been refined to reflect areas of either conserved or variable sequence among these channels.

Most of the models share certain features (Fig. 3) (Noda et al., 1984, 1986a; Greenblatt et al., 1985; Guy and Seetharamulu, 1986; Guy and Conti, 1990). With a single exception (Kosower, 1985), all explicitly recognize the presence of the four internally homologous repeat domains in the primary sequence and assume that these domains are organized in a similar fashion in the plane of the membrane. In view of the fact that all sodium channels lack an amino terminal signal peptide sequence, each model assigns the amino terminus to the cytoplasmic side of the membrane. With an even number of predicted transmembrane crossings per domain, the interdomain sequences and carboxy-terminus are given cytoplasmic locations as well. In addition, each of the models is constructed to optimize the

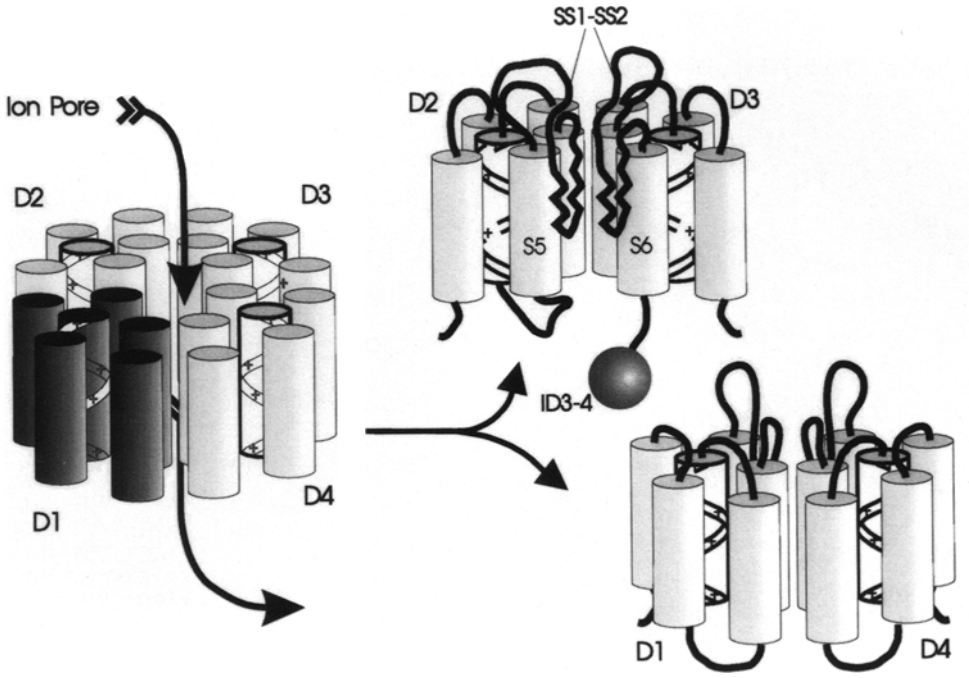

Fig. 3. Common features of proposed sodium channel tertiary structure. Most proposed structures for the sodium channel share certain common elements. The left part of the figure represents the organization of the transmembrane segments of the repeat domains. The right part illustrates the channel protein divided in half perpendicular to the plane of the membrane. Both the interhelical and the interdomain segments are shown as well. The repeat domains, each with six or more transmembrane helices, are compactly organized in the plane of the membrane. The charged S4 helices (shown in white) are in the interior of the domains. The domains surround, and contribute to the formation of, a central ion pore (left; contribution from one domain is shown in darker shading). Cytoplasmic and extracellular loops connect the helices within each domain. The loop between helix S5 and S6 is thought to extend down into the membrane to form the lining of the ion pore (right, upper half). The protein segments joining the repeat domains are all on the cytoplasmic surface of the protein. A special role is assigned to the segment joining domains 3 and 4 in channel inactivation; this region is shown as a ball in the figure.

number of potential cAMP-dependent protein kinase sites on the intracellular surface of the protein and to place the maximum number of consensus N-glycosylation sites on the extracellular surface. The models differ primarily in the details of the organization of the repeat domains and the structure of the ionic pore itself.

Noda et al. (1984) initially proposed that the four repeat domains were organized in a pseudosymmetric fashion within the plane of the membrane and that the weakly amphipathic S1 and S2 helices of each domain contributed to the formation of the central aqueous pore. The amphipathic S4 sequence was modeled as a 3^{10} helix with a vertical stripe of positive charge on a cylinder otherwise covered with nonpolar residues. The investigators assigned both the strongly and weakly amphipathic S4 and S3 helices to a cytoplasmic location.

Guy and Seetheramalu (1986) subsequently proposed that the amphipathic S4 α-helix of each domain lined the ionic pore. The positive charges of this α-helix were counterbalanced by negative charges in a region designated "S7," placed between helices S5 and S6 of each domain (Fig. 4). In this model, the S4 helix was proposed to be the voltage sensor and was placed within the potential gradient of the membrane. Channel

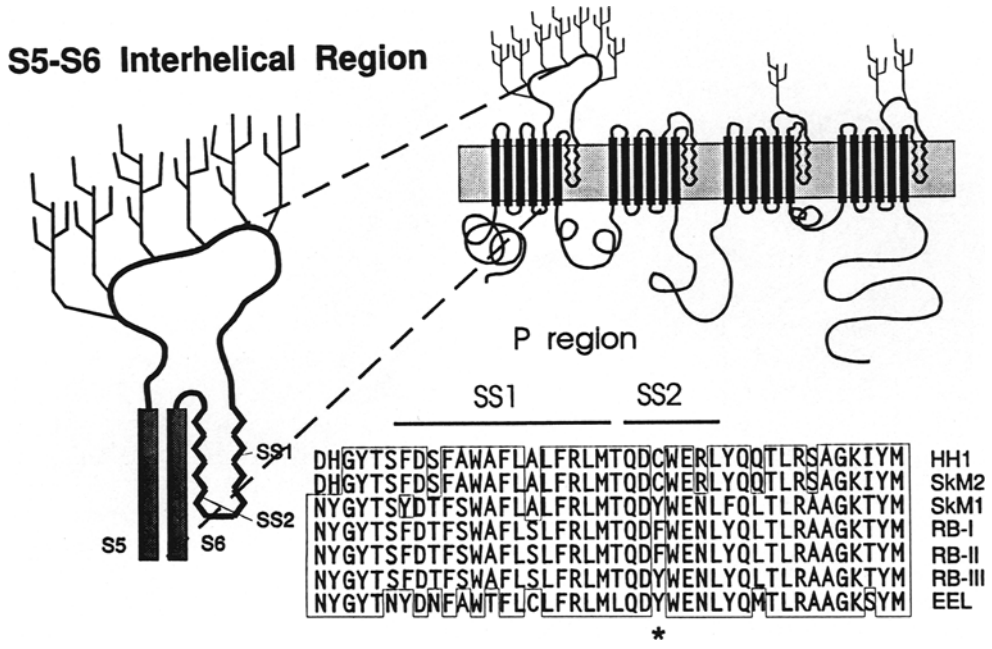

Fig. 4. Sequence conservation and proposed structure of the interhelical S5–S6/SS1 and SS2 regions. Part of the interhelical S5–S6 sequence may dip back into the membrane to form the lining of the central aqueous pore. This region is highly conserved (including conservative amino acid substitutions) and provides at least part of the binding sites for toxins that bind extracellularly to the mouth of the pore such as tetrodotoxin and saxitoxin. Interchange of a single amino acid from Y to C (position indicated by star) converts the channel phenotype from high affinity to low affinity tetrodotoxin binding.

activation gating was conceived as a screw-like motion of the S4 helices in one direction and S7 region in the other direction such that each positive charge becomes neutralized by the next lower negative side-group residue on the adjacent helix. By virtue of a 60° rotation in each helix, a 4.5 Å translocation of the S4 helices perpendicular to the plane of the membrane can be achieved with a concommitant net charge movement that could be detected as a gating current.

The model of Greenblatt et al. (1985), although similar to that described by Noda et al. (1984), presents several differences. The amphipathic S3 helices line the aqueous channel pore and the positively charged amphipathic S4 helices are transmembrane in orientation, placed in the interior of each of the homologous domains with S4 positive charges interacting with negative charges in S3 and S7. Two additional weakly hydrophobic sequences containing a number of charged residues (denoted SS1 and SS2, *see below*) were identified between the S5 and S6 helices of each domain; these were suggested to form additional membrane spanning structures. As in the model of Guy and Seetheramulu (1986), gating was proposed to involve movement of the S4 helix in response to changes in the transmembrane potential.

On the basis of new sequence information on sodium channels, two of these models have been updated. The rationale used was that sequences that were conserved between different sodium channel subtypes were likely to represent regions responsible for common channel function whereas variable regions might correspond to sequences that were either unimportant in channel function or that produce the unique characteristics of a particular channel isoform. Noda and his collaborators revised their original model by placing all six helical segments in each domain within the membrane (Noda et al., 1986a). The sequences lining the aqueous channel pore were

now provided by the S2 helix, which contain glutamic acid and lysine residues that are highly conserved in sodium channels from different tissues. In addition, the voltage sensing function was now attributed to the S4 helix which was placed in the interior of each repeat domain.

Guy and Conti (1990) have also proposed an updated model of the sodium channel that incorporates new sequence comparisons among cloned sodium channels as well as the results of experimental studies of channel topography. The S4 and S6 helices have been subdivided into amino-terminal regions (S4n and S6n), carboxyl-terminal regions (S4c and S6c), and, for the S4 helix, an α-helical mid-portion region (S4h). The SS1 and SS2 regions first described by Greenblatt et al. (1985) and referred to as the "S7" region by Guy and Seetheramulu (1986) are also preserved with minor modifications. Finally, the transmembrane region of the channel protein is divided into three concentric cylinders perpendicular to the membrane (interior, middle, and exterior) and into three cross-sectional layers parallel to the membrane (inner, middle, and outer). The exterior cylinder is comprised of the S1, S2, S3, and S5 helices of previous models and spans all three cross-sectional layers. The interior cylinder is composed of SS2 β-strands and S4c in either a β-strand or α-helical conformation and is present only in the middle layer of the three cross-sections, thus forming the narrow portion of an "hour-glass" shaped ionic pore. The middle cylinder of the middle layer is comprised of buried hydrophobic SS1 and S6n segments in an unspecified orientation. The large portals of the outer and inner layers (i.e., the entrance to and exit from the channel) are lined with segments that contain hydrophilic side chains: the extracellular approach is lined by S4n, S4h (in the open conformation), and segments between S5 and SS1 and between SS2 and S6, whereas the cytoplasmic exit from the channel is lined by the S4 to S5 loop and S6a. Charged groups near the ends of S1, S2, S3, and S5 are also proposed to be at least partially exposed to the intracellular lumen in the inner cross-sectional layer.

In the Guy-Conti model, voltage-dependent transitions between the open and the "deactivated" states involve conformational changes of only the S4 and SS2 segments and the sequences that link these segments to adjacent membrane-embedded sequences. In contrast to the "helical screw" or "sliding helix" model of activation gating, Guy and Conti propose a "propagating helix" model in which S4 has an extended or β-stranded structure at its amino- and carboxy-termini and assumes an α-helical conformation only in its midportion. Activation is associated with an amino-terminal movement of the α-helical structure such that the amino-terminus of S4 is transformed from an extended to an α-helical structure as the formerly α-helical mid-portion reverts to an extended structure, thus propagating the α-helix from middle cross-sectional region toward the outer cross-sectional region.

Experimental Studies

Common features of all current models of sodium channel structure include the presence of four membrane-embedded homologous domains, intracellular amino- and carboxy-termini, and intracellular sequences joining the homologous domains. The models diverge in the way in which the sequences within each repeat domain are organized and in the choice of sequences that line the aqueous pore of the channel. Despite the fact that many elements of these models (such as the overall structure of the protein, the role of the 3–4 interdomain region in channel inactivation, and the role of the S4 helices as voltage sensors) are appealing, the real worth of these models is that they generate predictions that can be tested and used to constrain the features of future models.

The most direct limits that can be placed on the postulated structure of the sodium channel protein have been provided by the localization of sites of posttranslational modification and by the identification of sequences comprising toxin or divalent metal binding sites. Since phosphorylation is an intracellular process, it is likely that the sequences phosphorylated in vitro by cAMP dependent pKA in channels from a variety of tissues (and by pKC in the case of the rat brain sodium channel) are restricted in their location to the intracellular surface of the protein.

Analyses of isolated eel electroplax, rat brain, and rat skeletal muscle sodium channel proteins have shown that all three channels are phosphorylated by the catalytic subunit of pKA to >50% of the maximal level of phosphorylation attainable if the channels are first stripped of phosphate; serine is the predominant acceptor residue. Seven 32P-labeled tryptic digestion fragments of the eel electroplax (Emerick and Agnew, 1989) and rat brain (Rossie et al., 1987; Rossie and Catterall, 1989) channels were obtained whereas similar treatment of the skeletal muscle channel yielded only one prominently labeled fragment (Yang and Barchi, 1990). The in vitro phosphorylation sites in the brain and skeletal muscle channels are located in the interdomain 1–2 region whereas those for the eel electroplax channel are found in the amino and carboxy ternini. In addition, sites of endogenous phosphorylation of the rat brain sodium channel by pKC have been localized to the ID 3–4 region (West et al., 1991). These studies restrict models of channel tertiary structure to those in which ID 1–2, ID 3–4, and the N-terminal and C-terminal segments are intracellular.

The localization of the sites of attachment of α-scorpion toxin photoaffinity derivatives to the rat brain sodium channel has placed additional constraints on the structure of the first homologous domain. α-Scorpion toxins are small polypeptides that bind to the extracellular surface of the sodium channel at Site 3 (Table 1) in a voltage-dependent manner and modifies channel activity by slowing inactivation. An azidonitrobenzoyl derivative of toxin V, the principal α-scorpion toxin from *Leiurus quinquestriatus* (LqTx), specifically photoaffinity labels both the α- and β_1-subunits of the rat brain sodium channels either in synaptosomes, intact neuronal cells in culture, or in reconstituted purified channel-containing lipid vesicles (Beneski and Catterall, 1980; Sharkey et al., 1984). Immunoprecipitation of a radiolabeled peptic fragment enabled the site of attachment of the photoactivatable group in the α-subunit to be localized within Domain I (Tejedor and Catterall, 1988). Additional proteolysis yielded a labeled but nonglycosylated 14 kDa cyanogen bromide fragment derived from within amino acids 313–426, requiring that the labeled site be located more toward the C-terminus than the five potential glycosylation sites in the proposed extracellular loop joining S5 and S6 in D1. This region overlaps with the SS1 and SS2 sequences that are postulated to form at least part of the transmembrane pore, also called the P-region (Fig. 4).

It is possible that this site of α-scorpion toxin attachment is merely adjacent to, rather than part of, the toxin receptor site, and a series of monoclonal and polyclonal antibodies were used in competition studies to further map the toxin binding site to residues 371–400 and 1686–1705, which are located in the region between the S5 and S6 helices of Domains 1 and 4, respectively (Thomsen and Catterall, 1989). The kinetics of toxin inhibition are consistent with direct competition between antibody and toxin for binding, and the possibility that decreased binding was owing to antibody-induced conformational changes propagated from distant regions in the protein was effectively ruled out. Scorpion toxins have a small binding surface consisting of only about 13 amino acids (Fontecilla-Camps et al., 1988), and the inhibition of toxin binding by antibodies to segments at opposite ends of the primary sequence suggests that they are closely associated in the tertiary structure (lower right panel, Fig. 4). In view of the fact that at least one of the β-subunits was also labeled by photoreactive toxin derivatives in earlier experiments, these results provide evidence for an extracellular compact toxin binding site composed of at least the S5–S6 loops of the first and fourth domains of the α-subunit in association with a region of the β_1-subunit. Since toxin binding interferes with the coupling of activation to inactivation (Thomsen and Catterall, 1989), it has been proposed that the toxin interacts with the S4 helices of both D1 and D4, interfering with the coupling of activation gating charge movements in Domain 1 to the conformational changes in Domain 4 that initiate closing of the inactivation gating segment.

The tertiary structure of the skeletal muscle sodium channel protein has also been probed by

limited proteolysis in conjunction with peptide segment-specific antisera. The channel protein was shown to be sensitive to endogenous proteases in regions predicted to link the four repeat domains, in particular the ID 2–3 region, whereas the repeat domains themselves were resistant to proteolysis (Kraner et al., 1989). When proteolyzed channel protein was probed with iodinated lectin, most of the carbohydrate was located between 22 and 90 kDa from the amino terminus of the channel, consistent with the observed clustering of consensus N-glycosylation sites in the first domain of all channels. These studies were extended using limited proteolysis with trypsin, chymotrypsin, and V-8 protease in conjunction with a more extensive panel of sequence-specific antisera (Zwerling et al., 1991). When the channel was in its native conformation either in membrane fragments or in mixed detergent-lipid micelles, cleavage occurred at a limited number of sites and in discrete, reproducible steps, suggesting a hierarchy of relative accessibility to these soluble enzymes. Since the order of appearance of the fragments was similar for all three enzymes, it was concluded that the observed pattern was determined by the accessibility of selected sites in the tertiary structure rather than by the amino acid sequence surrounding the cleavage sites. The carboxy-terminus of the protein was rapidly cleaved at multiple sites by each protease, whereas the amino-terminus proved remarkably protease-resistant. The order of cleavage was: C-terminus, ID 2–3, ID 1–2, and then ID 3–4. Although D1 and D4 appeared to remain intact throughout proteolysis, limit fragments for epitopes associated with D2 and D3 suggest that cleavage occurred later within the large loops at sites between the putative S5 and S6 helices in these domains. The amino terminus is most resistant to proteolysis and may assume a more compact globular structure in the native channel.

With knowledge of the sequence of the sodium channel and the location of the antibody epitopes and assuming that carbohydrate is distributed evenly at each glycosylation site, the carbohydrate content of each domain was estimated. The weight of the limit fragment arising from D1 suggests that most channel carbohydrate is covalently associated with this domain, supporting previous qualitative results with lectin binding to proteolytic fragments. The weight of the D2, D3, and D4 limit fragments were consistent with none, two, and one complex carbohydrate chain(s), respectively. Thus, the overall distribution of carbohydrate mass appears to conform to the distribution of potential N-glycosylation sites predicted from primary sequence analysis.

Sequence-specific antibodies can also provide information on the topological location of regions of the channel sequence when used to determine the orientation of their respective epitopes either in intact tissue or in vesicles containing oriented channels. Oligopeptides corresponding to residues 1781–1793 at the carboxy-terminus and residues 927–938 in the ID 2–3 of the eel channel primary sequence were injected into rabbits to yield segment-specific antibodies (Gordon et al., 1987, 1988). Antiserum and immunoaffinity-purified IgG against both of these peptides reacted only with a 260 kDa band on immunoblots of eel membrane protein and specifically labeled the innervated face of the eel electroplax at the light microscopic level. Secondary antibody labeled with colloidal gold was used to detect bound antibody at the electron microscopic level indicating that both antibodies were found exclusively on the cytoplasmic side of the innervated membrane bilayer. These studies localize the ID 2–3 region and the caboxyterminus to the cytoplasmic surface of the molecule and imply that, if the four repeat domains are homologous in organization, the protein must cross the membrane an even number of times in each domain.

This study was extended using monoclonal antibodies generated against purified rat skeletal muscle sodium channel protein. This panel of monoclonals was mapped both for location on the primary sequence and for binding interactions in the tertiary structure. Two groups of interacting epitopes were identified. The largest group included the binding sites for sixteen antibodies that were linked together through mutually competitive interactions (Kraner et al., 1989).

Localization of these epitopes using proteolyzed sodium channel demonstrated that several of the antibodies that were linked by competition studies were situated in different halves of the channel primary sequence (Cohen and Barchi, 1992a,c). Other epitopes for monoclonal antibodies that evidenced different histochemical staining patterns and were previously thought to distinguish between sodium channel subtypes in skeletal muscle were found to reside close together at the amino terminus of the same channel sequence, suggesting that the appearance of independent subtypes in tissue immunocytochemistry reflected the relative accessibility of these two epitopes, perhaps because of differential interactions of this structurally constrained region with β-subunits or with other membrane proteins (Cohen and Barchi, 1992a). An alternative possibility is that sodium channels encoded by the same gene exhibit differential splicing (Shaller et al., 1992).

Intracellular application of a polyclonal antibody raised to a short intracellular segment between D3 and D4 was shown to produce a gradual slowing of sodium channel inactivation in whole-cell recordings (Vassilev et al., 1988). This antibody-induced slowing of inactivation was greater during test depolarizations to more positive membrane potentials or when more negative holding potentials were used prior to the test pulse. The antibody did not alter single channel current amplitude, but channel openings were observed throughout the depolarizing pulse rather than being restricted to the onset of the pulse, consistent with the conclusion that this antibody blocked channel inactivation (Vassilev et al., 1989). Once again, these effects were more rapid in onset at more negative holding potentials and at more positive test potentials. These results suggest that the accessibility of the ID 3–4 segment is sensitive to membrane potential and that the antibody acts by interfering with the movement of this region. An alternative explanation would be an antibody-induced shift to an alternate gating mode characterized by prolonged open states and repetitive channel opening.

In Vitro Expression and Mutagenesis

The *Xenopus* oocyte expression system has provided electrophysiologists with a method for studying the function of protein products encoded by mRNA and cRNA for different sodium channel subtypes under controlled conditions. Employing either voltage clamp or patch clamp configurations, the unique characteristics of both native channels and channel mutants can be examined. Identification of sequences involved in a particular channel function is attained either by introducing specific mutations or through the formation and expression of channel chimeras. These approaches have proven useful in associating specific functions with specific regions of the primary structure.

Functional Expression

Poly-$(A)^+$ mRNA prepared from mammalian brain, heart, or skeletal muscle is capable of directing the synthesis of functional sodium channels when injected into *Xenopus* oocytes (Gunderson et al., 1984a,b; Hirono et al., 1985; Dascal et al., 1986; Sigel, 1987; Krafte et al., 1991). In several studies, size fractionation of crude mRNA preparations using sucrose gradient sedimentation yielded a single peak of large RNA transcripts that promote the expression of sodium channels in oocytes, implying that only the large α-subunit was needed for channel activity (Sumikawa et al., 1984). However, the expression of channels from nonhomogeneous populations of mRNAs is fraught with ambiguity.

Sodium channels have also been expressed in functional form in oocytes using synthetic cRNA encoding brain (Noda et al., 1984, 1986b; Stühmer et al., 1987; Joho et al., 1988; Suzuki et al., 1988; Ahmed et al., 1992), cardiac (Cribbs et al., 1990; Gellens et al., 1992), and skeletal muscle (Trimmer et al., 1989; Kallen et al., 1990; White et al., 1991; George et al., 1992b) sodium channel α-subunits. These results make it clear that the α-subunit of these sodium channels contains all the essential elements of fully functional channels

when expressed in oocytes. This conclusion, however, assumes that *Xenopus* oocytes do not produce endogenous β-type subunits or other elements that are essential for activity and capable of interacting with the exogenous α-subunit protein.

Although high molecular weight mRNA and cRNA derived from α-subunit cDNA directs the synthesis of functional sodium channels, the properties of these channels are often not normal. The time course of inactivation for many isoforms (e.g., rat brain and skeletal muscle subtype 1 channels) is much slower for α-subunits expressed in oocytes with cRNA than for channels expressed from total mRNA in mammalian cells or studied in vivo (Noda et al., 1986b; Trimmer et al., 1989; Joho et al., 1990; Krafte et al., 1990). The basis for the slow inactivation kinetics seen with the SkM1 muscle isoform in oocytes has been shown, from experiments at the single channel level, to be attributable to an increased frequency with which individual sodium channels shift from the characteristic kinetic mode with fast inactivation to a second mode characterized by altered inactivation with multiple late openings or channel reopenings (Zhou et al., 1992). Similar transitions have been observed in sodium channels in vivo in skeletal (Patlak and Ortiz, 1986, 1989) and cardiac muscle (Nilius, 1988), but at a much lower probability. The slow inactivation of macroscopic currents observed in oocytes expressing the SkM1 alpha subunit alone represents an increased frequency probability of this alternate kinetic mode rather than a constant change in the kinetics of channel inactivation. Repetitive or late channel openings have also been implicated in the abnormal behavior of sodium channels in several neuromuscular diseases to be discussed later.

Coinjection into oocytes of low molecular weight mRNA from rat brain (derived from nondenaturing sucrose-gradient fractionated poly-($[A]^+$ RNA) with the α-subunit cRNA reduced the time course of channel inactivation to values comparable to those seen in vivo and increased the cell surface expression of functional sodium channels almost fourfold (Auld et al., 1988). Moreover, when low molecular weight mRNA fractions or cRNAs prepared from a subgroup of cDNAs that do not themselves encode a sodium channel are coinjected with cRNA for the α-subunit, the equilibrium between normal and abnormal modes of inactivation *(see below)* is shifted to favor the normal mode (Auld et al., 1988; Zhou et al., 1992). The low molecular weight proteins programmed by mRNA or cRNA might represent sodium channel β-subunits, enzymes involved in posttranslational modification of sodium channel α-subunits, or other proteins, such as G-proteins or cytoskeletal elements, which interact with the sodium channel α-subunit.

Despite this range of possibilities, the abnormally slow activation kinetics of rat brain IIa expressed from cRNA in oocytes is corrected simply by coexpression of cRNA from the rat brain β_1-subunit. Not only is the voltage-dependence of inactivation shifted to more negative potentials, but the peak sodium current is increased (Isom et al., 1992). The slow inactivation of the SkM1 alpha subunit from rat or human muscle expressed in oocytes is also corrected by coexpression with either the rat β_1 subunit or the homologous human β_1 subunit (Bennett et al., 1993; Cannon et al., 1993; Yang et al., 1993). This acceleration in the kinetics of the macroscopic currents can be accounted for by a shift in the equilibrium between the two gating modes that is induced by the binding of the β_1 subunit. The increase in macroscopic current amplitude can also be accounted for on the basis of this shift in equilibrium between modes.

These results indicate that the β_1-subunit can normalize and modulate the kinetics of the α-subunit. However, the observation that pKC-dependent phosphorylation also slows channel inactivation suggests that protein phosphorylation/dephosphorylation may be another factor that contributes to the transition between gating modes (Numann et al., 1991). There may be a number of different mechanisms through which mode switching may occur in sodium channels. The relative importance of a single factor will certainly depend on the physiological conditions present at that time.

cRNAs for the TTX-insensitive form of the rat skeletal muscle sodium channel (SkM2), and for its homologous human heart sodium channel (hH1), program the expression in oocytes of sodium channels with normal inactivation kinetics (Fig. 5) (Gellens et al., 1992; White et al., 1991). Unlike SkM1, SkM2 and hH1 channels do not show late channel openings at the single channel level during depolarizing pulses. These channel isoforms may contain within them structural features that stabilize the normal kinetic mode of channel inactivation, or that mode may be stabilized by interactions with oocyte proteins that are unique to these channels.

Mutagenesis

Access to full-length cDNAs for various sodium channel isoforms has allowed molecular biological approaches involving deletions, insertions, mutations, and interchange of segments between isoforms to be used in order to define functional regions in the sodium channel primary structure.

Domain Structure

Some of the first experiments with sodium channels were motivated by the observation that potassium channels can be formed by the association of four identical but independently-translated subunits, each resembling a single sodium channel repeat domain (Zagotta and Aldrich, 1990; Stühmer et al., 1991). Might individual sodium channel domains or several contiguous domains interact to produce functional sodium channels in a manner analogous to these K^+ channel subunits? This was tested with cRNAs encoding the relevant regions of the rat brain II sodium channel coinjected into oocytes (Stühmer et al., 1989). Neither single domains nor transcripts that together contained up to three contiguous domains were capable of forming functional channels. Coinjection of cRNAs encoding D1 and D2:3:4 produced small sodium currents whereas the combinations of D1:2 with D3:4, D1:2:3 with D4, and a construct of D1:2:3:4 with deletions in the amino- and carboxy-termini produced functional channels, although the sodium currents were somewhat reduced compared to wild-type cRNA. These results suggest that all four domains are required to form functional sodium channels although they can assemble into channels even when the linkage between domains is interrupted.

Sodium channels containing deletions in the amino- or carboxy-termini as well as channels expressed from coinjection of D1:2 and D3:4 transcripts also demonstrated activation and inactivation kinetics comparable to wild-type channels. Channels produced by coinjection of the D4 and the D1:2:3 transcripts, however, demonstrated a dramatic slowing of channel inactivation. This was confirmed by measurements at the single channel level, which demonstrated channel reopenings throughout the voltage step. The slowing or loss of inactivation was similar to the effects reported for native sodium channels treated with proteolytic enzymes.

Channel Activation: S4 Helix

The role of the S4 helix in voltage-dependent activation was first evaluated by introducing point mutations, such as substitution of positively charged arginine or lysine residues by either neutral or negatively charged residues into the S4 regions of D1 and D2 (Stühmer et al., 1989). All single, double, and triple mutations produced functional channels although no expression of functional sodium channels was observed for any mutation that involved neutralization of more than three positive charges in S4 helices. A reduction in net positive charge in the S4 segment of D1 produced a decrease in the steepness of the voltage-dependence of channel activation that was directly proportional to the number of charges altered. A similar general correlation between positive charge in the homologous S4 helix and voltage-dependence of activation has been shown for mutated potassium channels (Lopez et al., 1991; Papazian et al., 1991) although the magnitude of the effects produced by charge neutralization varied greatly depending upon the residue modified. Since no functional channels have been obtained with charge neutralizing mutations of the central S4 residues, which are absolutely conserved among KCh, it may be that they play a crucial role in gating, perhaps because they experience the largest positional changes.

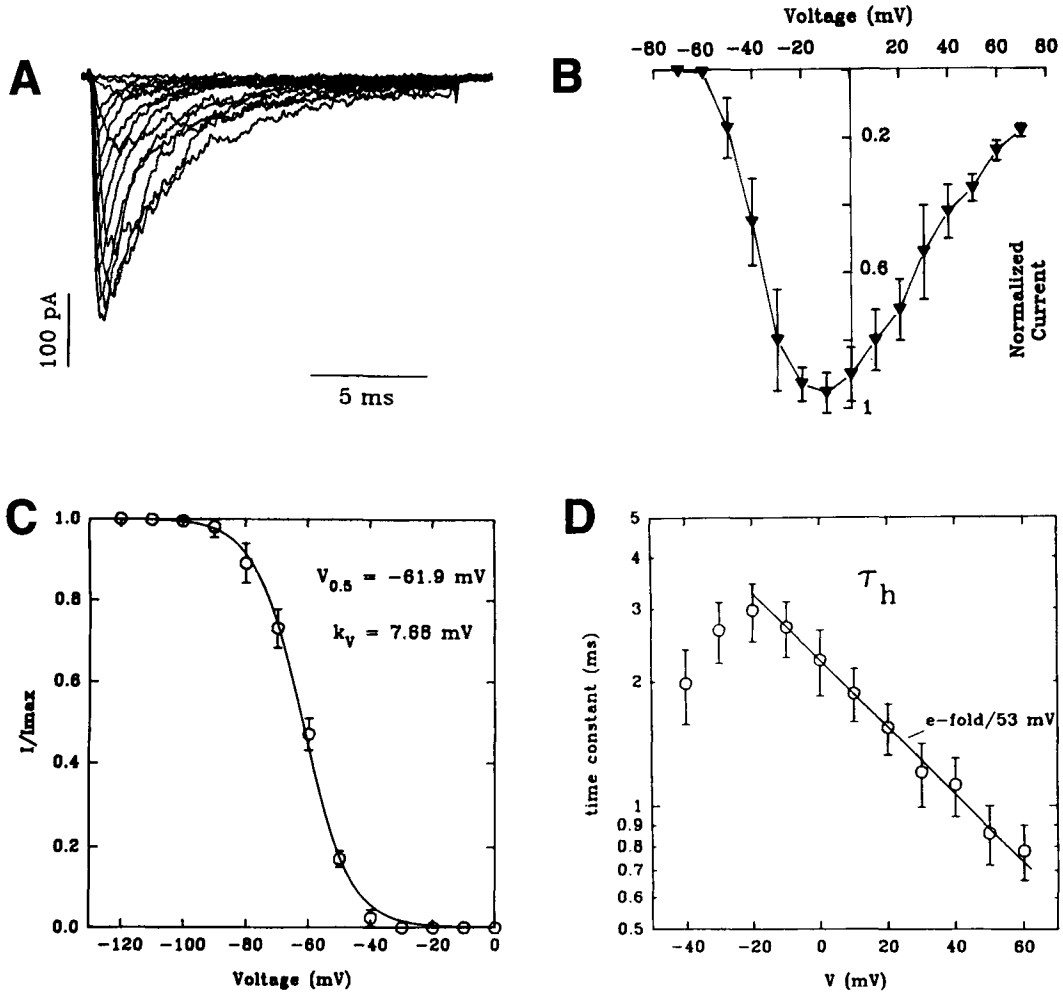

Fig. 5. Activation and inactivation of hH1 sodium currents expressed in oocytes. Full length cRNA encoding the rH1 channel was injected into oocytes and recordings were made from macropatches 2–3 d later. The basic kinetic properties of these human cardiac sodium channels are shown. (**A**) A family of voltage-clamp currents recorded from a large patch by depolarization from -120 mV to test voltages between -70 and +70 mV in 10 mV increments. (**B**) Normalized (± SD) peak I–V relationship for six patches. (**C**) The steady-state inactivation curve obtained from seven outside-out patches. (**D**) The voltage-dependence of T_h (from Gellens et al., 1992, with permission).

When charge conserving mutations were introduced into the possium channel (for instance, R377K), there was a reduction in the voltage dependence of activation. Since this occurs in the absence of a change in charge, it is unlikely that the mechanism of activation results only from an interaction between the S4 basic amino acids and the membrane potential (Papazian et al., 1991). On the basis of a related S4 mutational analysis in the RCK1 KCh, it has been suggested that the second, fourth, and sixth cationic groups interact with negative countercharges from other regions such as S2 (E225 and E235), S3 (D258 and E272), and the S5–S6 loop (D361 and D377) (Logothetis et al., 1992), stabilizing the closed channel conformation. The application of an electric field causes these ion pairs to break and the remaining S4 charges on the first, third, fifth, and seventh cationic side chains move to form new ion pairs, stabilizing the open conformation. The

intersubunit interactions in KCh gating appear to be nonindependent and cooperative (Tytgat and Hess, 1992).

These results on sodium and potassium channels provide strong experimental support for the involvement of S4 positive charges in the voltage sensing mechanism for channel activation. However, the complexity of such experiments is hinted at by the fact that in addition to changes in the steepness of voltage-dependence, some mutants produced significant shifts in the voltage dependence of activation. It is particularly intriguing that comparable voltage shifts in the activation curve have been found for mutations of uncharged residues in the S4 helix in which no change in charge occurred. This aspect of the role of the S4 helix in channel activation is derived from electrophysiologic differences between rat brain II and IIa channels, which differ at seven amino acid positions (Auld et al., 1990). Rat brain IIa transcripts produced channels in *Xenopus* with an activation current–voltage relationship shifted 20–25 mV in the depolarizing direction as compared to rat brain II, and also showed slow inactivation in whole-cell current recordings. As noted earlier, coinjection of low molecular weight rat brain RNA increased the speed of macroscopic inactivation to a rate comparable to channels formed from injection of total rat brain poly-$(A)^+$ RNA but had no effect on the altered current–voltage relationship. A systematic analysis of the seven amino acid differences between rat brain II and IIa revealed that a single neutral amino acid substitution of phenylalanine for leucine at residue 860 in the S4 helix of Domain 2 was responsible for the altered current–voltage relationship. The gene actually codes for leucine and not phenylalanine at this position, suggesting a cloning artifact in the rat brain IIa clone. This fortuitous occurrence provides further evidence that the alteration of neutral as well as charged residues in the S4 helix can affect the current–voltage relationship of channel activation, suggesting that factors other than side-chain charge can influence the voltage-dependent conformational transitions associated with channel activation. It is interesting, however, that effects on gating valence have not been shown for any noncharged residues (Logothetis et al., 1992), including mutations of as many as 10 noncharged residues simultaneously.

Similar conclusions have been drawn from experiments with potassium channels, where systematic mutations of neutral residues in S4 to other neutral amino acids differing in size had significant effects on both the rate and voltage dependence of channel activation. These studies suggest that activation does not depend simply on the net charge on a protein dipole, but rather reflects the various energy levels characterizing the closed, intermediate, and open states contributed to by interactions of nonpolar residues within and between helices (Papazian et al., 1991). Such nonelectrostatic interactions may have little tolerance for change if coordinated charge movements are to be retained.

Taken as a whole, the analysis of mutations introduced into the S4 segment of NaChs and KChs supports the view that this segment comprises at least part of the voltage sensor for these channels (Liman et al., 1991; Logothetis et al., 1992; Papazian et al., 1991; Stühmer et al., 1989).

Channel Inactivation: ID 3–4

Mutations introducing breaks in the ID 3–4 greatly reduce channel inactivation (Stühmer et al., 1989). Additional support for assigning the role of channel inactivation to the ID 3–4 region comes from related studies on potassium channels in which site-directed mutagenesis was combined with the deletion of selected regions of the amino terminus (Hoshi et al., 1990). Basing their studies on the "ball and chain" model of channel inactivation (Armstrong and Bezanilla, 1977), these investigators demonstrated that the amino terminus acted like a ball tethered to the membrane-embedded domain by a chain, the length of which could, within certain limits, be altered without affecting channel inactivation. Changes in the size or charge of the distal amino terminal "ball" resulted in the loss of inactivation, which could be restored by the addition of millimolar amounts of a soluble synthetic peptide corresponding to the first 20 amino acids of the amino terminus (Zagotta et al., 1990). Thus, fast inactivation is produced by the amino terminus of

potassium channels in a manner that may be analogous to the proposed function of the sodium channel ID 3–4 region located immediately N-terminal to Domain 4. In fact, an N-terminal sodium channel ID 3–4 segment is able to restore inactivation to an inactivation-minus potassium channel (Patton et al., 1992b).

The ball and chain model of channel inactivation has gained considerable support from the above studies and from the knowledge that the 11 lysines and 1 arginine in the ID 3–4 region might be targets for endopeptidases that remove channel inactivation. Positively charged residues present on the ball were predicted to interact with negatively charged residues on the inner end of the pore. Thus, removing positive charges from the ball might be expected to delay channel inactivation without affecting channel activation. To test this prediction, groups of two or three adjacent lysines (six of the twelve possible) in the rat brain III ID 3–4 were replaced with neutral or negatively charged amino acids (Moormann et al., 1990). Contrary to the above prediction, inactivation occurred more rapidly in mutant channels with neutralized charges rather than more slowly. Reversal of charge at one location (R1461E), however, did result in a channel with delayed activation. Although many of the cationic side chains were not necessary for fast inactivation, the charge modifications in these experiments may have altered the electrostatic interactions of the ID 3–4 linker with other parts of the channel protein, indirectly affecting inactivation. An alternate explanation is that the mutation might cause a shift in the distribution of channels between two gating modes to favor the gating mode with faster inactivation.

Rapidly inactivating currents are recorded in oocyte-expressed mutant rat brain sodium channels with 11 of 12 basic amino acids and all 3 acidic amino acids substituted with glutamine, indicating that most of the charged amino acids in the ID 3–4 loop are not essential for inactivation (Patton et al., 1992a). However, one deletion mutant and a triple mutation (IFM → QQQ) completely removes fast inactivation and the single mutation (F1489Q) removes most of the fast inactivation (West et al., 1992). Clearly, the ID 3–4 region and particularly hydrophobic residues have more complex effects on sodium channel gating than previously thought. This type of inactivation-minus channel provides an ideal substrate for experiments to probe the characteristics of the inner mouth of the channel pore.

Permeation: Pore Structure and Blockers

Potassium Channels

Mutagenesis experiments involving KChs and their interactions with blockers (charybdotoxin {CbTX} and tetraethylammonium ions {TEA}) point strongly to the markedly conserved S5–S6 segment (also designated H5, SS1–SS2, or P loop) being intimately involved in the pore structure. The S5–S6 linker in Shaker KChs has 40 amino acids, including two basic and four acidic side chains. One of the current suggestions is that the SS1–SS2 segment exists as a β-sheet which turns back on itself to form a hairpin within the membrane and associates in a fourfold symmetric fashion with the S5–S6 loops of other subunits forming an eight-stranded β-barrel (Yool and Schwarz, 1991).

Mutations in the SS1–SS2 loop of the Shaker KCh affect the binding of a 37-amino acid polypeptide blocker from scorpion venom, CbTX (15 × 15 × 25 Å, net charge of +4) (MacKinnon and Miller, 1989). Since occlusion by extracellular CbTX is antagonized by extracellular TEA and external TEA block is cleared by K^+ ions entering from the cytoplasmic side, the bound toxin appears to interact broadly with the outer vestibule of the channel while extending into the mouth of the pore to plug it. Consistent with this view is the voltage-dependence of CbTx block (Anderson et al., 1988). The observation that elevated ionic strength reduces the block and results of site-directed mutation are both consistent with the conclusion that electrostatic interactions are important for CbTX binding. Thus, two mutants, E422Q and E422K, that make the charge on the side chain progressively more positive, result in a decrease in affinity of CbTX, consistent with electrostatic forces being important for CbTX binding (MacKinnon and Miller, 1989). The effects on CbTX binding of mutations K427R,

K427N, and K427E were interpreted to implicate electrostatic interactions with the 427 site closer to bound CbTX than the 422 site rather than direct (e.g., ionized H-bond) (MacKinnon and Yellen, 1990). In contrast, a second set of mutations, D431, T449, and V451, which affect CbTX block but do not involve a change in charge, clearly indicate that more than electrostatic interactions are involved, perhaps direct interactions involving ionized H-bonds (MacKinnon and Yellen, 1990).

Probing the KCh with a smaller molecule, TEA, support the hypothesis that the S5–S6 region contributes to the formation of the ion permeation pathway. TEA blocks KChs from the extracellular side with different affinities depending on the KCh subtype and the nature of the mutations. Substitutions of amino acids segregated, once again, into two classes. The D431K mutations cause changes in TEA block consistent with an electrostatic mechanism but E422Q, E422K, and K427E mutations had little effect on external TEA action, despite their large effects on the binding of the larger CbTX molecule as noted above (MacKinnon and Yellen, 1990). A key position is T449 (at the junction of the S5–S6 loop and the S6 transmembrane segment), since T449K, T449V, and T449Q all show a reduced or removed sensitivity to external TEA (MacKinnon and Yellen, 1990) as is seen in studies of the RCK4 KCh (Stühmer et al., 1989). In contrast, the T449Y mutant (tyrosine is also present at the homologous position in the highly TEA-sensitive drk1 [RCK1] mammalian KCh homologs [Salkoff et al., 1992]) exhibits a decreased IC_{50} of >50-fold for TEA (MacKinnon and Yellen, 1990). Finally, K, R, and Q substitutions for T449 exhibit loss of inward rectification and decreased single-channel current amplitude leading to the conclusion that the conduction pathway was altered in these mutants (Yellen et al., 1991). Further experiments support a mechanism in which an external TEA-binding site is formed by a bracelet of pore-lining aromatic residues enabling cation-π orbital interaction (Heginbotham and MacKinnon, 1992).

A complementary series of experiments used TEA to probe the cytoplasmic surface of the Shaker KCh and indicated that T441S determines internal TEA block sensitivity (Yellen et al., 1991). The T441S mutant shows increased sensitivity to internal TEA and the voltage-dependence of block indicated that the TEA site sensed 15% of the field from inside to outside. If T449 is on the outside as models predict, then the segment T441 to T449 (nine amino acid residues) traverses 80% of the membrane field. To be consistent with the actual distance, a β-strand (2.7 nm) rather than an α-helix (1.2 nm) seems more likely and, as had been concluded earlier, the pore is much shorter than the bilayer thickness (Hille, 1992).

Mutations of the S5–S6 loop of the Shaker KCh affecting ion selectivity were positioned at F433S, F433Y, T441S, and T442S with NH_4^+ and Rb^+ conductances increased, and that of K^+ unaltered. The mutant KChs retain the ability to discriminate against Na^+ (Yool and Schwarz, 1991).

The drk1 and NGK2 differ in phenotypes in several respects. The latter has single channel conductance threefold higher and sensitivities to external and internal TEA of 20-fold higher and 100-fold lower, respectively. A chimera with 24 residues of the S5–S6 loop of NGK2 inserted into the drk1 background exhibited the NGK2 phenotype, suggesting that these pore characteristics are determined solely by this 24 amino acid stretch (Hartmann et al., 1991). Point reversions in the NGK2 segment to the amino acids present in drk1 were consistent with the idea that the segment between P430 and P450 (using numbering system of Shaker H4 clone [Yool and Schwarz, 1991]) is deep within the pore and nonpolar valine and/or leucine residues at sites 438 and 443 suggest that nonpolar residues may be more important than charged or polar side chains in forming the deep part of the pore.

Sodium Channels

When Domain 1 NaCh sequences are aligned with those of KChs, the position of E387, which is important for TTX and STX block and will be discussed below, falls within three or four residues of the 449 site in the KChs that affects TEA and CbTX block in KChs. This suggests that these blockers probably bind at structurally homologous positions and that determining how the blocker binding site relates to the pore mouth for any given channel may shed light on the structure of all voltage-sensitive channels.

Because there was evidence from pH and trimethyloxonium experiments in which single channel conductance, sensitivity to external Ca^{2+}, and TTX was altered (Hille, 1992), investigators examined the consequences of removing carboxylate groups in the S5–S6 loop. Two such residues were found in the SS2 region of the first domain, two in the SS2 region of the second domain, and one in the SS2 region of the fourth domain (the SS2 segment of D3 is devoid of negatively charged amino acids). Neutralizing the charge of a single glutamic acid residue located in the P loop of domain 1 (D1), the E387Q mutation in rat brain subtype II NaCh, greatly reduced toxin sensitivity and reduced the channel conductance of a TIX-S channel expressed in oocytes, a finding identical to that obtained with carboxyl-modifying reagents (Noda et al., 1989). Such a reduction in inward current might be the result of a decreased concentration of sodium ions near the ion pore consistent with a localized decrease in negative charge owing to the E387Q mutation. Conformational changes propagated through the molecule by the primary sequence change are also possible, but it seems more likely that this glutamic acid residue is in close proximity to the mouth of the channel and is involved either directly or indirectly in toxin binding and ion permeation.

An extension of these studies involved mutations of homologous, primarily charged, residues in the carboxy-terminal segment of the S5–S6 loop in each of the four repeat domains of the rat brain II channel, many of which produced changes in TTX affinity (Terlau et al., 1991). These findings suggest that portions of all four S5–S6 loops contribute to the formation of the TTX/STX binding site. Furthermore, several of these mutations produced drastic reductions in channel ionic conductance without affecting gating currents, supporting a role for this portion of the primary sequence in the formation of the channel ion pore (Kontis and Goldin, 1993; Terlau et al., 1991). These investigators concluded that toxin binding involves more than electrostatic interactions and that these sites probably involve the permeation path as well. A model with two clusters of anionic residues forming ring structures at the extracellular mouth and/or pore wall of the NaCh was proposed to account for the effects of these mutations on toxin binding and permeation (Terlau et al., 1991).

Two of the mutants examined in the series mentioned above, K1422E(D3) and A1714E(D4), demonstrated reduced selectivity for Na^+ and bi-ionic reversal experiments with K1422E, and the double mutant showed behavior much like voltage-dependent calcium channels (Heinemann et al., 1992).

Surprisingly, when the sequence of the TTX-I sodium channels from rat and human heart and rat denervated muscle became available for comparison with those of TTX-S channels, all of the negatively charged residues noted earlier were conserved, except for Asp^{1545} in the SS2 region of Domain 4, which is Tyr^{1728} in the TTX-I channel sequences. Specifically, Glu^{387} was present in all the TTX-I channel primary sequences, clearly indicating that the difference in affinity between TTX-S and TTX-I channels does not involve this residue.

A different approach to localizing the residues connected with toxin binding utilized channel chimeras in which the first domains of the TTX-S and TTX-I rat muscle channels were interchanged. Channel chimeras composed of $(D1)_{SkM1}$ $(D2:3:4)_{SkM2}$ and $(D)_{SkM2}$ $(D2:3:4)_{SkM1}$ were constructed and expressed in oocytes (Chen et al., 1992). The TTX-phenotype of the chimeric channel segregated with the origin of the first domain. An extension of these experiments involved interchanging 22 amino acid peptides in the SS2 segment of the S5–S6 loop in the first domain; once again the TTX-phenotype of the chimera corresponded to that of the parent molecule from which the segment originated. Site-specific substitutions of a single amino acid in one isoform by that present in the other isoform (total of six amino acid differences, two within the SS2 segment) localized the difference in TTX-sensitivity to a *single* amino acid residue, namely Y^{401} (TTX-S) $\rightarrow$ C^{374} (TTX-I) in SkM1 and the reciprocal mutation C^{374} (TTX-I) $\rightarrow$ Y^{401} (TTX-S) in SkM2/rH1 (Chen et al., 1992; Satin et al., 1992; Backx et al., 1992). An aromatic amino acid (Tyr or Phe) in the carboxy-terminal region of the

S5–S6 loop of Domain 1, a segment previously modeled to form part of the central aqueous pore, confers the TTX-S phenotype whereas a cysteine at this position yields a TTX-I sodium channel. A favorable interaction between aromatic side chains and the cationic TTX molecule, similar to interactions proposed for TEA in K^+ channels (Yellen et al., 1991), is postulated to account for the increased affinity for TTX in TTX-S channels (Chen et al., 1992). The presence of cysteine at this site also is associated with sensitivity to Cd^{2+} block (Backx et al., 1992).

Therefore, in both KChs and NaChs the S5–S6 region seems crucially involved in determining the ion selectivity of the permeation pathway.

Regulation of Sodium Channel Gene Expression

Excitable membranes contain a number of sodium channel isoforms that are encoded by separate genes. There are at least four distinct transcripts expressed in rat brain, two in rat skeletal muscle, and as many as five in rat heart (Auld et al., 1988; George et al., 1992b; Kallen et al., 1990; Kayano et al., 1988; Noda et al., 1986a,b; Rogart et al., 1989; Schaller et al., 1992; Sills et al., 1989; Trimmer et al., 1989; Gautron et al., 1992). The mechanisms that regulate the induction and tissue-specific expression of these different isoforms are poorly understood. Studies in tissue culture have identified a variety of extra- and intracellular signals that affect the expression of sodium channel subtypes, including electrical activity (Yang et al., 1991), thyroid hormone (Brodie and Sampson, 1989), cytosolic sodium concentration (Dargent and Couraud, 1990), cyclic nucleotide levels, and a variety of channel active drugs and toxins (Taouis et al., 1991a,b). Serum and growth factors and cellular or viral oncogenes have also been shown to alter sodium channel expression (Caffrey et al., 1989; Estacion, 1990; Flamm et al., 1990; Kalman et al., 1990). The *jun* and *fos* proto-oncogenes may be involved in transsynaptic regulation of muscle-specific gene expression that occurs following surgical denervation (Bessereau et al., 1990).

In this section, we will review some of the more physiologically oriented studies and then describe recent work directed at understanding the molecular mechanisms responsible for the regulation of sodium channel subtype expression.

Brain Sodium Channels

The ontogenesis of sodium channels in the central nervous system has been examined using action potential recordings, neurotoxin-binding assays, sodium flux measurements, and, more recently, subtype-specific antibody and nucleic acid probes. Rather sparse developmental information was available until subtype-specific probes derived from cloning studies were used to quantitate rat brain subtype mRNA levels during development (Beckh et al., 1989; Beckh, 1990). The three rat brain sodium channels exhibit different temporal and regional patterns of expression in the developing rat central nervous system. Quantitation of mRNA levels indicates that in the adult rat the brain type II isoform is expressed at the highest levels whereas type I is present at approx 5–10% of these levels. Type III mRNA is expressed at moderately high levels in embryonic brain, but is barely detectable in the adult (Beckh et al., 1989). In terms of localization, rat brain I mRNA is expressed predominantly at late postnatal stages in the caudal regions of the brain and in the spinal cord, rising to adult level following the second and third postnatal weeks. Rat brain II mRNA reaches adult levels after the first or second postnatal weeks and is preferentially expressed in rostral rather than caudal regions of the brain and spinal cord. In the cerebellum, rat brain II transcripts increase from low levels postnatally to high adult levels. Rat brain II, therefore, appears to be expressed throughout development but with considerable regional variability. Rat brain III mRNA is expressed diffusely in fetal and early postnatal stages but the amount drops shortly after this period to variable but low levels in the adult (Beckh et al., 1989).

Many factors alter the surface expression of sodium channels in nervous tissue. Experiments

on the biosynthesis and processing of sodium channels in developing neuronal tissues have shown that most newly synthesized sodium channel α-subunits are not disulfide linked to β_2-subunits but exist in a metabolically stable intracellular pool, readily available for rapid membrane insertion (Schmidt et al., 1985). In these studies the definition of the site of control was not determined and it is not yet apparent whether transcriptional events, translational mechanisms, or transport to the cell surface is the limiting step. Increases in surface sodium channel protein expression during neuronal development were initially measured by neurotoxin binding (Berwald-Netter et al., 1981; Jover et al., 1988) and $^{22}Na^+$ influx assays (Couraud et al., 1986). Sodium channels were predominantly located on the cell bodies and axon hillocks of neurites (Boudier et al., 1985; Angelides et al., 1988).

Among the factors involved in the selective induction of brain sodium channels, nerve growth factor (NGF) has been, perhaps, the best studied. PC12 is a pheochromocytoma-derived cell line that can be induced to differentiate into sympathetic-like neurons by NGF. In the absence of NGF, PC12 cells are electrically inexcitable, but following several weeks of NGF treatment, morphologic differentiation and the development of rapid-upstroke action potentials are observed. It was early recognized that the expression of sodium channels in these cells best accounted for the ability of differentiated PC12 cells to generate action potentials.

Using subtype-specific mRNA probes, selective induction of the rat brain II channel was observed following treatment with NGF; no effect on rat brain I expression was found (Mandel et al., 1988). Measurements of $^{22}Na^+$ in the presence of sodium channel activators yielded similar results, with NGF-induced increases in TTX-S channels (presumably rat brain II) in addition to TTX-I channels (Rudy et al., 1987). Neurite outgrowth and sodium channel expression, as shown by whole-cell voltage clamp, was induced in these PC-12 cells by exposure to either NGF or basic fibroblast growth factor (FGF) (Pollock et al., 1990). FGF induced the same or somewhat larger increases in channel density when compared with NGF but produced less neurite outgrowth. Epidermal growth factor, on the other hand, produced no neurite outgrowth but did induce a small increase in sodium channel density. Pharmacologic manipulations that increase in intracellular concentrations of cyclic AMP levels did not stimulate neurite outgrowth but did result in a decrease in the amount of functional sodium channels. Lastly, dexamethasone was found to inhibit the increase in sodium channel expression produced by NGF but did not inhibit the effect of NGF on PC12 neurite outgrowth. Thus, sodium channel expression parallels the changes in morphology that lead to neurite outgrowth but does not depend on them and it appears that these two aspects of neuronal differentiation are independently regulated by factors in the microenvironment.

Since NGF is believed to affect neurite outgrowth in part through activation of cAMP dependent protein kinase A (pKA) and phospholipid-dependent protein kinase (pKC), agents that stimulated each of these systems were examined for effects on sodium channel expression using patch-clamp of PC12 cells (Kalman et al., 1990). Compounds that activate pKC (including phorbol esters and a *ras* oncogene product [p21]) stimulated neurite outgrowth but produced no effect on channel number. In contrast, agents that increased intracellular cAMP were about equally effective, as was NGF, in affecting both neurite outgrowth and channel expression, indicating a role for pKA in the effects of NGF. These results differ from the results of Pollock et al. (1990) mentioned above. Athough the reasons for this disparity remain unclear, different techniques (whole-cell clamp vs patch clamp) and experimental conditions may account for the disagreement.

The role and sites of action of pKA in modulating sodium channel expression have been clarified by studies of PC12 cell lines deficient in pKA activity (Ginty et al., 1992). In lines deficient in both pKA isozymes, the expected increase in functional sodium channel density in response to NGF is absent by whole-cell patch clamp analysis but Northern blot and STX-binding assays of intact cells confirm that increases in channel

mRNA and protein still occur. Thus, pKA appears to affect posttranslational expression of functional sodium channels in PC12 cells.

Additional studies shed light on the role of intracellular signal transducers in sodium channel expression. AtT-20 cells, derived from a mouse anterior pituitary tumor, express both TTX-S and TTX-I sodium channels. Transfection of activated *ras* into the AtT-20 cell line suppressed the expression of TTX-I channels but had no effect on the expression of TTX-S sodium channels (Flamm et al., 1990). Similarly, the fusion of two cell lines, which expressed either activated *c-Ha-ras* (human bladder carcinoma EJ cell line) or TTX-I sodium channels (human fetal lung fibroblast GM2291 cell line), created a fusion line with unaltered *c-Ha-ras* and no sodium channel expression (Estacion, 1990). These observations suggest a role for the *ras* protein in the regulation of expression of at least the TTX-I sodium channel.

Attempts to identify the genetic elements involved in the cell-specific expression of the rat brain II sodium channel gene have used transient expression assays (Maue et al., 1990). Chimeric reporter constructs composed of progressively smaller portions of a 1051-bp 5' rat brain II flanking sequence fused with a sequence encoding the bacterial enzyme chloramphenicol acetyltransferase (CAT) were constructed and expressed in several cell lines. CAT expression in neuronal cells but not in muscle-derived cells, fibroblasts, or HeLa cells, was consistent with the presence of elements within the 5' region that confer cell-specific expression upon the rat brain II gene. 5' deletion studies indicate that the 134-bp promoter region is not sufficient to confer cell specificity. These investigators identified at least three upstream elements that appear to provide neural-tissue specificity.

Additional deletion studies revealed a 28-bp silencer element, designated RE1, in the 5'-flanking region of the rat brain II gene, which confers neural-specific transient expression of CAT fusion genes (Kraner et al., 1992). RE1 did not resemble any of the negative elements that have been described for other genes, including the insulin, immunoglobin heavy chain, α-fetoprotein, myosin heavy chain, and SkM2 sodium channel genes, indicating that unique transcriptional factors are likely to be involved in the cell-specific expression of the rat brain II gene. Gel shift assays indicate RE1-binding protein factors in fibroblasts, skeletal muscle cells, and a neuronal cell line that is devoid of rat brain II channel expression. RE1 is similar to NRSE (neural-restrictive silencer element) from the SCG10 gene and may be one of a family of *cis*-elements controlling neuron-specific expression in vertebrates. The repression by RE1 and NRSEs might involve displacement or inactivation of some positive-acting transcription factors (Levine and Manley 1989). Alternatively, repression may simply reflect the net effects of negative- and positive-acting regulatory elements involving gain of silencer-binding factors or loss of "anti-repressors," or some combination of both (Mori et al., 1992).

Skeletal Muscle Sodium Channels

Alpha Subunits

Pharmacologic studies have demonstrated that mammalian skeletal muscle expresses at least two forms of sodium channel differing in TTX-sensitivity (Barchi and Weigele, 1979; Rogart and Regan, 1985). The sodium channel in adult skeletal muscle is TTX-sensitive (TTX-S), whereas the channel synthesized in embryonic and denervated muscle and in heart is relatively insensitive to TTX (TTX-I). The development of the adult phenotype in mammalian skeletal muscle requires the interaction of muscle fibers with active motor neurons since TTX-I currents appear within 2 d of denervation (Harris and Thesleff, 1971; Pappone, 1980; Redfern and Thesleff, 1971). Although high affinity STX-binding sites in the sarcolemma decrease over the first 2 wk following denervation to approx 60% of baseline values (Weigele and Barchi, 1982; Bambrick and Gordon, 1988), the level of SkM1 mRNA changes little during this period (Yang et al., 1991) suggesting that some regulation of SkM1 expression may take place at the posttranscriptional level. Single-fiber voltage-clamp measurements indicate that at least 40% of the channels in denervated muscle are of the TTX-I subtype (Pappone, 1980).

The distribution of voltage-dependent sodium channels on the postsynaptic side of the mouse neuromuscular junction is maximum on the edge of the synaptic gutter: There are no Na^+ channels on fold crests. The crest border integrates postsynaptic potentials and generates the muscle action potential (Boudier et al., 1992). Studies with isolated motor endplates from mouse intercostal muscles showed colocalization of the nicotinic receptor and of the voltage-dependent Na^+ channel (Dreyfus et al., 1986).

A number of factors have been implicated in the regulation of expression of skeletal muscle sodium channels in tissue culture. Down-regulation of channels is induced by the sodium-ionophores, amphotericin B and monensin, or by batrachotoxin in a matter of hours (Dargent and Couraud, 1990; Bar-Sagi and Prives, 1985) and is consistent with channel internalization rather than decreased channel synthesis. In the case of batrachotoxin-induced down-regulation, the process was reversible and required protein synthesis.

The role of neuromuscular activity in the cell-surface expression of sodium channels in muscle was investigated using radiolabeled toxin binding to neonatal rat muscle (Bambrick and Gordon, 1988). In the course of normal development, channel expression increased exponentially with a doubling time of 12 d. Most rapid channel incorporation coincided with the period of accelerated muscle growth and neuromuscular activity at 2 wk of age. When neuromuscular activity was eliminated, both muscle growth and the normal incorporation of sodium channels were retarded. Denervation was more effective than treatment with botulinum toxin during development, while both were equally effective in reducing channel number in adult tissue, implying different modes of control of sodium channel expression during development and in the adult.

Further insight into the intracellular mechanisms responsible for neuromuscular control of sodium channel expression comes from studies of electrical activity, intracellular calcium concentration, cAMP, thyroid hormone, and treatment with verapamil or ethanol on the modulation of channel expression in skeletal muscle cells in culture.

The administration of thyroid hormone produces a dose-dependent increase in sodium channel expression which correlates with increases in action potential frequency and rate of rise of the action potential in cultured skeletal myotubes (Brodie and Sampson, 1989). The effect is rapid, with onset at 12 h and plateau levels are attained by 36–48 h. Both inhibitors of protein synthesis and increased extracellular calcium concentration block or reduce this increase. Lowering intracellular calcium concentrations by treatment with TTX or verapamil produces a marked increase in thyroid-stimulated sodium channel synthesis.

In related studies, the normal age-dependent increase in channel expression in cultured muscle cells was decreased by preventing myoblast fusion (Brodie et al., 1989). Sodium channel down-regulation occurred following treatment with the calcium ionophore A23187, and up-regulation was induced by eliminating spontaneous electrical and contractile activity with TTX, elevated external KC1, or verapamil. Since protein synthesis inhibitors blocked channel up-regulation, it was concluded that electrical and mechanical activity, exerting their influence through alterations in cytosolic calcium, regulate the *de novo* synthesis of sodium channels.

Additional evidence that intracellular calcium may play a role in regulating the expression of skeletal muscle sodium channels comes from detailed studies of the effects of verapamil and ethanol on cells in culture (Offord and Catterall, 1989; Brodie and Sampson, 1990, 1991). Inhibition of spontaneous electrical activity with local anesthetics (bupivicaine) or elevated intracellular cAMP concentration produced a twofold increase in sodium channels in rat skeletal muscle myotubes measured with radiolabeled toxins (Brodie and Sampson, 1989; cf. rat cardiac myocytes, Taouis et al., 1991b) whereas treatment with A23187 decreased sodium channel density and overcame the effect of blocking electrical activity. Using a rat brain II cDNA probe, a parallel increase in message and channel protein expression was revealed both during development and following treatment with bupivicaine or 8-Br-cAMP, indicating that the control of α-subunit mRNA levels is the primary mechanism

of regulation of sodium channel density by electrical activity in rat muscle cells.

Subtype-specific mRNA levels in adult muscle, denervated muscle, and muscle treated with botulinum toxin were analyzed with subtype-specific nucleic acid probes (Yang et al., 1991). Different mechanisms appear to be involved in the regulation of the expression of mRNAs encoding TTX-S (SkM1) and TTX-I (SkM2) sodium channels. In innervated muscle, TTX-I transcript expression is suppressed and TTX-S channel expression is considerably higher in fast twitch than slow twitch fibers. Withdrawal of electrical stimulation, whether through surgical or chemical denervation, has little effect on TTX-S mRNA levels, suggesting that the decline in density of high affinity TTX binding sites in denervated sarcolemma may reflect regulation at the translational or posttranslational rather than transcriptional levels. SkM2 (TTX-I) mRNA expression, however, is rapidly induced when electrical activity is eliminated (Fig. 6), and, based on studies with botulinum toxin, its level of expression appears to reflect nonquantal release of factor(s) from the motor nerve terminal ending (Yang et al., 1991). Quantal acetylcholine release, on the other hand, may play a major role in suppressing TTX-I sodium channel expression in innervated or reinnervated muscle.

The influences of growth factors on developing skeletal muscle cells have also been investigated. NGF and FGF caused dose-related increases in sodium channel expression as assessed by both STX binding and measurement of the frequency and rate of rise of spontaneously occurring action potentials (Offord and Catterall, 1989). Although both fused and unfused C2 mouse skeletal muscle cells possess TTX-S sodium channels, treatment of these cells with transforming growth factor β_1, an inhibitor of myogenic differentiation, inhibits the expression of TTX-S channels. Mitogen withdrawal allows sodium channel reexpression (Caffrey et al., 1989). Thus, growth factors appear to have similar effects on both nerves and skeletal muscle cells in culture. Additional study of the detailed mechanisms and the level at which control is exerted is clearly necessary.

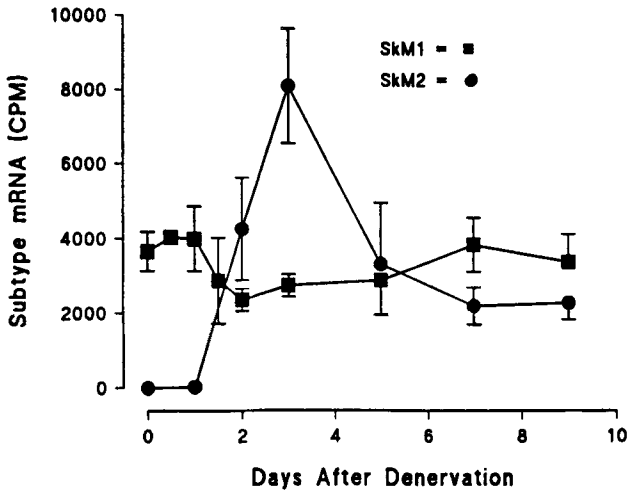

Fig. 6. Time course of changes in SkM1 and SkM2 mRNA expression after axotomy. The sciatic nerve was transected in the mid-thigh region in rats. SkM1 mRNA declined early by a small but significant amount but returned to baseline values by d 7. SkM2 mRNA, undetectable between 0 and 24 hours, rose rapidly to a peak at 72 h after axotomy before declining to a steady state intermediate level (from Yang et al., 1991, with permission).

The *cis*-regulatory elements and transcription factors that provide transcriptional regulation of the TTX-I sodium channel are also being examined (Sheng et al., 1993). Transient expression of 5' upstream elements driving the CAT reporter gene in muscle and nonmuscle cultured cells have identified a skeletal muscle cell-specific core promoter and at least one positive and one negative *cis*-regulatory element. In addition, four GC-rich elements (Sp1 recognition sites), four overlapping C-rich motifs believed to be important for muscle-specific expression of certain genes (Sartorelli et al., 1990) and one MyoD helix-loop-helix protein binding site immediately upstream of the core promoter were identified (Sheng et al., 1993). A comparison of upstream elements for rat brain II and SkM2 genes shows no detectable homology. The cell type specific expression of the TTX-I sodium channel appears to be regulated in a complex manner by multiple interactions of gene-specific positive and negative *cis*-acting elements.

Beta Subunits

As reviewed, encoding the beta 1 subunit have been cloned from rat brain (Isom et al., 1992), heart (Bennett et al., 1993), and skeletal muscle (Yang et al., 1993) as well as from human brain (McClatchey et al., 1993) and skeletal muscle (Makita et al., 1993). The cDNAs from each of the rat sources encode identical proteins whereas the human cDNA is highly homologous to the rat both in the coding region and in the 5' untranslated region.

Beta 1 mRNA is expressed in high levels in brain, heart, and skeletal muscle in the adult; lower levels are detectable in kidney and uterus as well (Yang et al., 1993). These investigators also studied the regulation of beta 1 mRNA expression in skeletal muscle. Beta 1 transcripts are barely detectable by Northern analysis in neonatal muscle but increase progressively during postnatal development in a pattern that parallels the expression of the SkM1 alpha subunit (Fig. 7). SkM2 mRNA, on the other hand, shows reciprocal regulation with highest levels in the neonate that decline to undetectable by 2–3 wk postnatal. In adult muscle, beta 1 mRNA levels also change in parallel with SkM1 rather than SkM2. After denervation, when SkM2 is upregulated by more than 100-fold, there is no significant change in beta 1 mRNA.

Measurements of beta 1 expression have also been made in primary cultures of rat skeletal muscle cells (Yang et al., 1993). Beta 1 mRNA is expressed at very low levels in myoblasts; expression increases during fusion and subsequent development of myotubes, roughly in parallel with both SkM1 and SkM2. However, blockade of electrical activity with TTX, which dramatically increases the expression of SkM2 mRNA, has no effect on either beta 1 or SkM1 mRNA. Likewise, artificial elevation of intracellular cAMP increases SkM2 message expression but has little effect on either beta 1 or SkM1.

Although the mechanisms of beta 1 regulation remain to be defined, it is clear that this subunit mRNA is expressed in parallel with the SkM1 alpha subunit under a variety of developmental and physiological conditions.

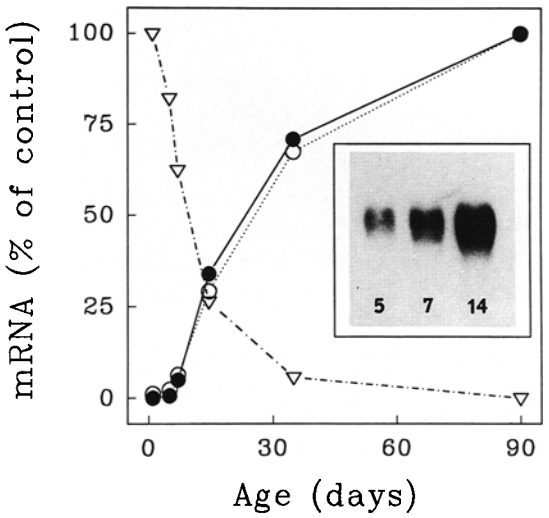

Fig. 7. Expression of SkM1, SkM2, and beta-1 mRNA during development in rat skeletal muscle. Steady-state levels of mRNA encoding the SkM1 and SkM2 isoforms of the channel alpha subunit and the beta-1 subunit were determined in rat skeletal muscle at the indicated times after birth. The level of SkM2 mRNA is highest at birth and declines rapidly during the first few weeks of life. In contrast, both SkM1 and beta-1 mRNA levels are virtually undetectable at birth but rise progressively and in parallel to maximal levels in adult muscle. Northern blots of beta-1 mRNA at d 5, 7, and 14. Beta-1 mRNA is also coregulated with SkM1 rather than SkM2 after denervation in adult muscle and in primary muscle in culture (from Yang et al., 1993).

Cardiac Sodium Channels

Toxin and electrophysiological studies have indicated the existence of both TTX-S and TTX-I sodium channels in heart (Catterall and Coppersmith, 1981; Rogart et al., 1983; Renaud et al., 1983; Tanaka et al., 1984; Rogart and Regan, 1985). Although rat brain channels subtypes I and II are expressed in cardiac tissue, at least three nominally cardiac-specific channels have been cloned and sequenced (Kallen et al., 1990; George et al., 1992a; Sills et al., 1989). The distribution and density of the channel isotypes in different regions has not been reported. However, the tools are now available to accomplish this and to study numerous influences or paradigms with potential affects on cardiac excitability:

1. α_1- or β-adrenergic stimulated hypertrophy (Bishopric and Kedes, 1991);
2. Hypercontraction of chemical hypoxia {cyanide or deoxyglucose} (Bond et al., 1991);
3. Metabolic impairment by iodoacetamide (Buja and Willerson, 1991);
4. Block of intercellular communication by volatile anesthetics (Burt and Spray, 1989);
5. Calcitonin gene-related peptide {CGRP} increased beating frequency (Chatterjee et al., 1991);
6. Effect of KC1 depolarization {G-proteins} (Foster et al., 1990);
7. Phorbol ester stimulation (Dunnmon et al., 1990; Henrich and Simpson, 1988);
8. Doxorubicin induced myopathy {myofibrilar loss} (Ito et al., 1990);
9. Stretch-length dependence (Komuro et al., 1990);
10. Sympathetic innervation by coculturing with thoracolumbar sympathetic ganglia (Lloyd and Marvin, 1990);
11. Angiotensin II (Moorman et al., 1989);
12. Thyroid and glucocorticoid hormone (Orlowski and Lingrel, 1990);
13. Fibroblast and transforming growth factors and myotrophin (Parker et al., 1990; Sen et al., 1990).

Regulation of cardiac channel expression or function by cAMP, G-proteins, class I antiarrhythmic drugs and atrial natriuretic factor has been reported but detailed mechanistic studies are just beginning (Sorbera et al., 1993; Sorbera and Morad, 1990, 1991; Schubert et al., 1989; Yatani et al., 1990; Matsuda et al., 1992; Zhang et al., 1992; Taouis et al., 1991a).

Sodium Channels and Disease

Chromosomal Localization of Sodium Channel Genes

The human cardiac (hH1), skeletal muscle (hSkM1), and brain (subtype II) sodium channels have now been cloned, sequenced, and functionally expressed (Gellens et al., 1992; George et al., 1992b; Ahmed et al., 1992). Using species- and subtype-specific probes, hamster–human somatic cell hybrid lines have been screened to determine chromosomal localization of the cognate genes. hH1 has been localized to chromosome 3 whereas hSkM1 is on chromosome 17 (George et al., 1991). Employing a chromosome 17 hybrid panel, the hSkM1 gene (SCN4A) was further localized to the region between 17q23. 1 and 17q25.3 (George et al., 1991).

Using comparable techniques and *in situ* chromosomal hybridization, the gene encoding the human counterpart of the rat brain II sodium channel was localized to chromosome 2 between 2q21 and 2q24.3 (Litt et al., 1989; Han et al., 1991; Ahmed et al., 1992). Multiple copies of this gene (SCN2A) or several related genes might be present within this region. Mouse brain sodium channel genes may be organized in a similar fashion since homologs of the rat brain I, II, and III genes have been mapped to a contiguous small segment of the mouse chromosome 2 (Malo et al., 1991). The genes for the mouse homologs of SkM1 and SkM2 have been located on mouse chromosomes 11 and 9, respectively, the latter being isotenic with human chromosome 3 (Ambrose et al., 1992; Klocke et al., 1992).

The coding region for hSkM1 in the human gene, SCN4A, is contained in a 32.5-kb segment of chromosome 17 and consists of 24 exons (54–2242 bp in length) and 23 introns (97–4850 bp) (McClatchey et al., 1992; George et al., 1993). There is no apparent relationship of the exons to predicted functional domains of the protein; some splice sites fall in the middle of presumptive transmembrane helices (Fig. 8). The exon–intron boundaries in the human gene correspond precisely to those contained within an 8-kb homologous segment of the rat gene and 10 of 24 splice junctions in SCN4A are positioned in homologous locations in the *para* gene in *Drosophila*, which encodes another putative sodium channel (George et al., 1993).

The Human Periodic Paralyses and Paramyotonia Congenita

Pathophysiology

The chromosomal localization of the human muscle sodium channels has been used in linkage studies of several neurologic diseases in which abnormalities in sodium channel function

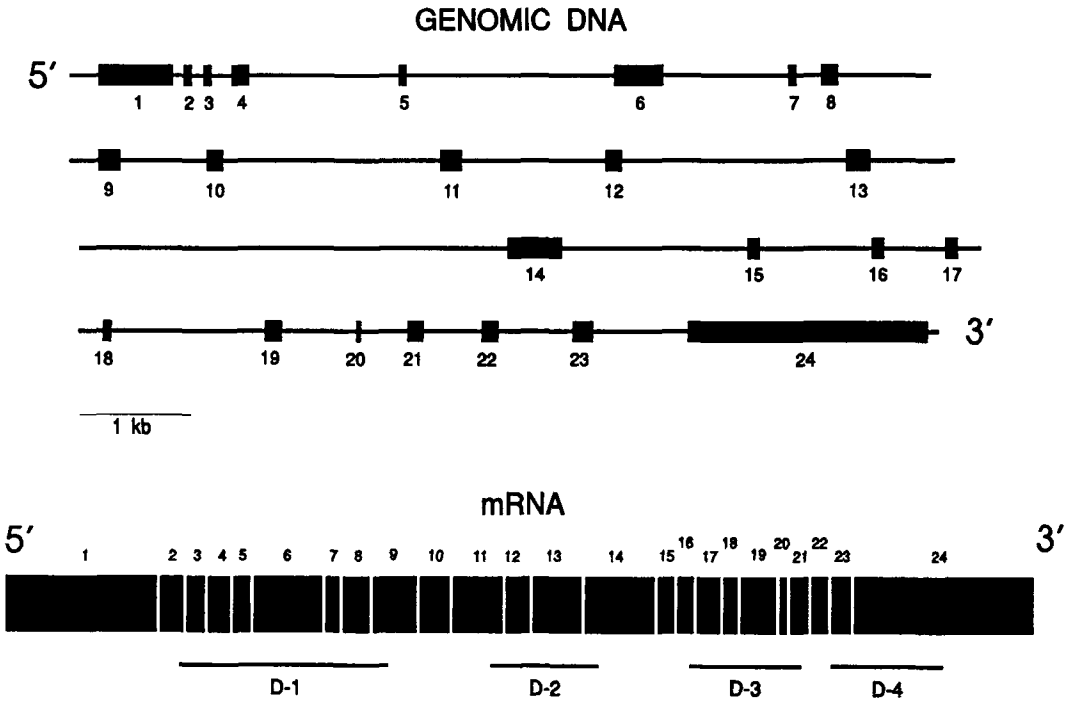

Fig. 8. Organization of the human SkM1 sodium channel gene at 17q21.1–23.5. The coding region, contained within 32.5 kb of genomic DNA, consists of 24 exons and 23 introns. Although the positions of the exon–intron junctions seem to fall in homologous locations in those sodium channel genes that have been analyzed, these junctions do not correspond to functional divisions within the channel primary sequence.

are thought to play a role in disease pathophysiology. The periodic paralyses constitute a group of rare disorders affecting skeletal muscle that are characterized by intermittent episodes of weakness or paralysis, often with apparently normal neuromuscular function between attacks. Paralytic episodes can be triggered by specific dietary factors such as a large carbohydrate load, by environmental factors such as cold exposure, or by physiological changes such as rest after vigorous exercise. Despite the fact that there are some recognized acquired forms, most disorders in this category are inherited in an autosomal dominant fashion. Occasionally the paralytic phenotype is associated with signs of membrane hyperexcitability in the form of myotonic discharges and stiffness (myotonia).

Depolarization of the muscle sarcolemma accompanies the paralytic episodes seen in these unusual disorders (Rudel et al., 1984; Lehmann-Horn et al., 1987a,b). When the skeletal muscle of afflicted persons has been studied in vitro, the depolarization is the result of an abnormal increase in membrane permeability to sodium ions. In hyperkalemic periodic paralysis (HyPP) and paramyotonia congenita (PC), the abnormal sodium conductance can be blocked by TTX, implicating the voltage-dependent sodium channel as the defective protein (Lehmann-Horn et al., 1987a,b; Ricker et al., 1989). Voltage-clamp recordings in HyPP demonstrate a small noninactivating sodium current that is present only with elevated extracellular potassium and appears to be responsible for the persistent membrane depolarization that characterizes the paralytic episodes (Lehmann-Horn et al., 1987a).

Single channel recordings have been made on cultured myotubes from a HyPP muscle biopsy (Cannon et al., 1991) and support the hypothesis that a primary sodium channel abnormality underlies the pathophysiology of this disease. With normal extracellular potassium, the single channel kinetics of sodium channels in HyPP appear normal. An increase in $[K^+]_{out}$ to 10 m*M*

had no effect on control channels, but caused a small percentage of the HyPP sodium channels to exhibit aberrant gating properties. An intermittent loss of inactivation, with multiple channel openings during a depolarizing pulse, and bursts of late channel openings were observed with these channels. In prolonged single channel recordings, abnormal inactivation was temporally clustered, indicating that the defective channels were able to switch between normal and abnormal kinetic modes. The failure of inactivation seen in HyPP channels with high potassium is consistent with the non-inactivating sodium current previously reported in voltage clamp studies.

Linkage Analysis

Analysis of linkage between the SCN4A gene encoding the hSkM1 channel on chromosome 17 with the phenotypic expression of hyPP was carried out in several well-characterized families with this disorder to test the involvement of this channel in the pathogenesis of the disease. A restriction fragment length polymorphism (RFLP) in the region encoding the ID 2–3 was employed to demonstrate significant linkage between the SCN4A gene locus and a phenotype of HyPP with myotonia (Fontaine et al., 1990). A different *Bg*lII RFLP was used to show comparable linkage in another family expressing HyPP without myotonia (Ebers et al., 1991). Studies of additional HyPP families with or without myotonia confirmed linkage to the SCN4A sodium channel gene (Ptácek et al., 1991b; Koch et al., 1991). Linkage to SC4NA was also established by analyses on families with the paramyotonia congenita phenotype (Ptácek et al., 1991b; Ebers et al., 1991). Thus, these two muscle disorders are allelic, both being associated with defects in the SCN4A gene. An atypical form of painful myotonia congenita has been linked to the SCN4A gene (Ptácek et al., 1992b).

Identification of Sodium Channel Mutations

In order to identify specific mutations in the SCN4A gene in HyPP, the known cDNA sequence for hSkM1 was used to identify exon–intron boundaries in the genomic DNA (George et al., 1993). Based on this information PCR primers for individual exons were generated and employed to screen genomic DNA from seven unrelated affected individuals for mutations by analysis of single-strand conformational polymorphism (Ptácek et al., 1991a). A cytosine to thymidine mutation was identified in three of these individuals that resulted in the substitution of a methionine residue for an absolutely conserved threonine at position 704 in S5 of Domain 2. This amino acid substitution segregated with affected members of two families, and appeared as a spontaneous mutation in the third. The defect was not found in either of the parents of the third patient, in unaffected members of the other families, or in any of 109 controls.

An alternative approach was employed to identify mutations in two other cases of HyPP (Rojas et al., 1991). In this study, mRNA was prepared from a muscle biopsy of an individual with HyPP. cDNA produced from this mRNA was then analyzed to define an adenine to guanine mutation that results in the substitution of a methionine for a highly conserved valine^{1592} in S6 of Domain 4. This mutation was shown to segregate with disease expression in the affected members of this family and to appear as a new mutation in another affected family (Ptácek, 1991a, 1992a).

Two separate mutations affecting the same codon were subsequently identified in three unrelated families with paramyotonia congenita (Ptácek et al., 1992a). These mutations, which substitute either histidine or cysteine for Arg^{1448}, neutralize conserved positive charge in the S4 helix postulated to be involved in channel voltage-dependent activation. Two other mutations have been found in PC that affect conserved amino acids near the N-terminus of the ID 3–4 loop, a region that has been implicated in channel inactivation (G1306V and T1313M).

In a separate PC family, a T to G transversion was identified that introduces an arginine for a conserved leucine^{1433} near the extracellular end of the S3 helix in D4 (Ptácek, 1992a). This mutation will produce a major change in local charge and in sidechain packing at a region of the channel physically adjacent to the two S4 mutations.

Two additional mutations have been identified in HyPP and PC families with symptoms that overlap traditional diagnostic categories (McClatchey et al., 1992). One pedigree, with features of PC and HyPP, has an alanine^{1156} to threonine amino acid change in the predicted intracellular loop between S4 and S5 in D3. The second pedigree exhibits features of PC and myotonia congenita; this family expresses a serine^{804} to phenylalanine mutation near the cytoplasmic end of the S6 helix in D2.

Periodic paralysis within a quarterhorse lineage has also been linked to a sodium channel mutation. In these animals, a phenylalanine^{1363} to leucine mutation in D4 S3 has been identified. This residue is thought to lie in the transmembrane domain near the cytoplasmic face of the membrane (Rudolph et al., 1992).

Thus, it appears that a variety of point mutations in the sodium channel gene are capable of producing diseases with related phenotypes (Fig. 9). Formal analysis of each will be needed to determine the mechanisms through which the mutations act, but these experiments of nature will undoubtedly prove valuable in further analyzing channel structure and function. We anticipate, furthermore, that mutations in cardiac sodium channels will form the basis for diseases with altered excitability in the heart.

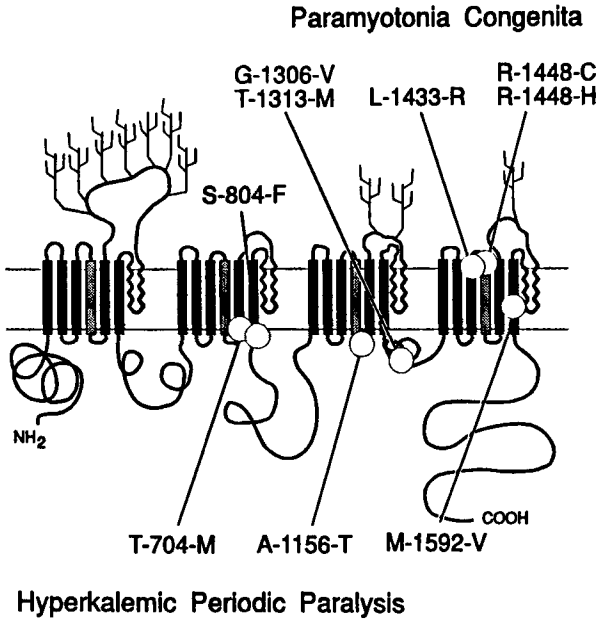

Fig. 9. Summary of point mutations identified in families expressing the hyperkalemic periodic paralysis or paramyotonia congenita phenotype. Mutations associated with the hyperkalemic periodic paralysis phenotype are mostly located near the cytoplasmic ends of domain helices in D2 and D4. Paramyotonia congenita mutations are located in the ID3–4 loop, in the D4 S4 helix, and in the D3 S3 helix immediately adjacent to it. The latter three mutations introduce changes in charge of the affected residues.

Expression of Channels Containing Disease Mutations

Several of the mutations that have been identified in these human diseases have been recreated and expressed in vitro either in the rat SkM1 or the human SkM1 background. Cannon et al. (1993) introduced a T^{698}–M mutation comparable to that described by Ptácek et al. (1991b) and a M^{1585}–V mutation homologous to the one identified by Rojas et al. (1991) into the rat SkM1 wild type background. The rat and human channels exhibit absolute conservation of sequence in the regions of the D2S5 and D4S6 helices in which these mutations occur, and the hypothesis was that the effects of these mutations would be similar in the rat and human counterparts of this channel isoform. These investigators reported that both mutations disrupted inactivation without interfering with the timecourse of channel activation, leading to persistent noninactivating currents. At the single channel level, the T^{698}–M mutation showed reopenings or very late first openings, and prolonged channel open times. The M^{1585}–V construct showed more normal kinetics in most depolarizations, with occasional complete loss of inactivation and high opening probability throughout the depolarization. Single channel conductance was not affected by either mutation. Neither of the mutant channels showed changes in kinetics when extracellular potassium was increased.

A second group reported slightly different results with the same T^{698}–M mutation expressed in the rat SkM1 background (Cummings et al., 1993). Although these investigators also found an increase in the appearance of abnormal inacti-

vation events in their voltage-clamp records, they felt that the frequency of occurrence (3% for the mutant channel vs 1% for the wild-type) was insufficient to explain the phenotype of the disease. They reported a shift in the voltage-dependence of activation by 10–15 mV in the negative direction which they conclude could underlie the abnormal muscle activity seen in these patients.

The two mutations identified in the D4S4 helix in families with paramyotonia congenita have also been expressed in both the human and the rat SkM1 homologs (Chahine et al., 1993). Neither mutation (R^{1448}–H and R^{1448}–C in the human sequence) affected the activation of whole cell currents in a human kidney cell line transiently transfected with either the rat or the human constructs. However, both mutations in either construct resulted in channels that inactivated more slowly with less voltage-dependence than the wild-type. Single channel analysis of the human mutants showed that the channels inactivate normally from closed stated but poorly from the open state. This was especially striking in the R^{1448}–C mutation, where it appears that an open channel cannot inactivate directly. These data suggest a critical role for the D4S4 helix in the coupling of activation to inactivation. Neither of the mutant constructs in the rat or human background showed potassium sensitivity, and little difference was noted in the temperature dependence of their kinetics of inactivation.

Conclusion

In the span of four decades, the sodium channel has been purified, reconstituted, sequenced, functionally expressed, and selectively mutated. Our understanding of the molecular mechanisms responsible for the complex behavior of this large protein are being revealed using a combination of molecular, immunologic, and protein chemical techniques. Amino acid sequences involved in channel activation, inactivation, gating, voltage sensitivity, and toxin binding have been identified. Several axioms that underlie sodium channel structure–function relationships are evolving. It is likely that continued application of these approaches and the addition of imaging methods for large complex membrane proteins will provide an even more detailed and complex model of sodium channel tertiary structure and function. These findings, when coupled with a better understanding of how sodium channels contribute to the pathophysiology of disease processes in nerve, skeletal, and cardiac muscle, will lead to new approaches to diagnosis and therapy.

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