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Cellular and Molecular Biology of Voltage-Gated Sodium Channels

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I. INTRODUCTION

The voltage-sensitive sodium channel is responsible for the increase in sodium permeability during the initial rapidly rising phase of the action potential in nerve, neuroendocrine, skeletal muscle, and heart cells. Like most cells, these electrically excitable cells maintain a high intracellular K^+ concentration and a low intracellular Na^+ concentration relative to the extracellular fluid through the energy-dependent pumping of these cations by the Na^+-K^+-ATPase. Excitable cells also maintain a resting membrane potential, inside negative, because their surface membranes are specifically permeable to K^+. Electrically excitable cells are distinguished, however, by having voltage-sensitive ion channels in their surface membranes that respond to membrane potential changes with large regenerative increases in permeabilities to specific ions on a time scale of milliseconds. Changes in ionic permeability are correctly measured only if the membrane potential of the cell is controlled experimentally. The landmark papers of Hodgkin and Huxley (122–125) and Hodgkin, Huxley, and Katz (126) introduced the voltage-clamp technique and

provided a remarkably complete analysis of the changes in ion permeability occurring on the millisecond time scale that are responsible for generation of the action potential in the squid giant axon. The depolarizing phase of the action potential was found to be due to an increase in sodium permeability, whereas the repolarization and hyperpolarization following the action potential spike were due to an increase in potassium permeability.

The voltage-clamp experiments also showed that the sodium permeability increase resulting from depolarization of nerve or muscle cells is biphasic. On depolarization, sodium permeability first increases dramatically and then decreases to the baseline level after ~1 ms. This biphasic behavior was described in terms of two experimentally separable processes that control sodium channel function: activation, which controls the rate and voltage dependence of the sodium permeability increase following depolarization, and inactivation, which controls the rate and voltage dependence of the subsequent return of sodium permeability to the resting level during a maintained depolarization. The sodium channel can therefore exist in three functionally distinct states or groups of states: resting, active, and inactivated. Both resting and inactivated states are nonconducting, but channels that have been inactivated by prolonged depolarization are refractory unless the cell is repolarized to allow them to return to the resting state. These fundamental properties of sodium channels established in the initial studies of Hodgkin and Huxley (122–125) and Hodgkin, Huxley, and Katz (126) have been the foundation on which essentially all subsequent studies of sodium channel function have been based.

II. MOLECULAR PROPERTIES OF SODIUM CHANNELS INFERRED FROM FUNCTIONAL STUDIES

Studies of sodium channel function in intact excitable membranes using voltage-clamp methods have led to important inferences about sodium channel structure that provide essential background for design and interpretation of current efforts to probe the molecular basis of sodium channel function. In this section, a few notable examples of functional studies using the voltage-clamp technique that led to valuable working models (Fig. 1) of the structure of functional elements of the sodium channel are briefly considered.

A. Ion Conductance and Ion Selectivity Filter

Sodium channels are remarkably efficient in ion conductance. The ion conductance of a single sodium channel has been estimated indirectly from voltage-clamp experiments and measured directly by the patch-clamp method. Comparison of voltage-clamp currents with measurements of sodium channel density by saxitoxin or tetrodotoxin binding (6, 151; see sect. III) in squid giant axons or frog muscle fibers gave a unit conductance of 2.5–8.6 pS. Analysis of voltage-dependent membrane current fluctuations due to sodium channel activation using Fourier transform methods yielded an estimate of 4.1–8.8 pS (66, 67, 244) in squid giant axon and frog node of Ranvier. The original direct measurements by single-channel recording gave an estimate of 12–18 pS (177, 245) at 20°C and 140 mM Na^+. The previous estimates by other methods gave smaller values because sodium channels open only briefly on depolarization so that not all channels are open at once and because current fluctuations systematically underestimate peak single-channel current values. Nevertheless, all these estimates imply physiological ion transport rates $>10^7$ ions/s, consistent with the movement of Na^+ through a fixed pore or channel. These rates can be compared with turnover numbers for enzymes, which range up to 5×10^5 s^{-1} for carbonic anhydrase. Ion conductance mediated by sodium channels is among the most rapid protein-mediated processes.

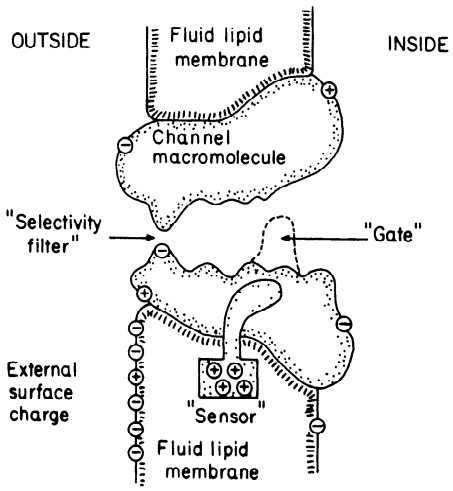


FIG. 1. Model of sodium channel based on electrophysiological studies using voltage-clamp method. This drawing summarizes conclusions concerning functional components of sodium channels from biophysical studies using voltage-clamp method. Model depicts a transmembrane pore with a small external vestibule and a large internal vestibule, a selectivity filter that interacts with permeant ions, an inactivation gate on intracellular side of pore, and positively charged voltage sensors that initiate voltage-driven conformational changes leading to channel activation in response to membrane depolarization. [From Hille (120a).]

In addition to being unusually rapid, ion conductance by the sodium channel is selective. Potassium is ~8% as permeable as sodium, and rubidium and cesium are even less permeant (65). Hille (118, 119) extended these early measurements to additional metal cations and a large number of organic cations and developed a model of the narrowest region of the ion-conducting pore of the sodium channel, the ion selectivity filter. On the basis of the selectivity of the pore for organic cations, the limiting region was proposed to be approximated by a 3.1 × 5.1 Å rectangular orifice lined by oxygen atoms, which act as hydrogen bond acceptors during transport of organic cations and hydrated metal cations but which exclude similarly sized ions having

non-hydrogen-bonding substituents like methyl groups. The transport of ions through the activated sodium channel is blocked by protonation of one or more acid groups with an acidic dissociation constant (pK_a) of ~5.2 (117). On the basis of this finding, it was proposed that two of the oxygen atoms acting as hydrogen bond acceptors at the ion selectivity filter are the oxygens of carboxylic acid groups that are required in the deprotonated form for effective ion transport. The selectivity of cation transport parallels the binding of these cations at a high field strength ion-exchange site (119). Because the carboxylate anion(s) postulated to be a required constituent of the selectivity filter is a high field strength site, interaction of partially hydrated metal cations with this site was proposed to provide the basis of selectivity among metal cations.

B. *Evidence for a Voltage-Dependent Conformational Change Associated With Sodium Channel Activation*

The changes of functional state resulting in activation are due to voltage-dependent conformational changes in a protein component(s) of the sodium channel. On theoretical grounds, a membrane protein that responds to a change in membrane potential must have charged and/or dipolar amino acid residues located within the membrane electrical field. Changes in the membrane potential then exert a force on these protein-bound dipoles and charges. If the energy of the field charge interactions is great enough, the protein may be induced to undergo a change in conformation to a new stable state in which the net charge or the location of charge within the membrane electrical field has been altered. For such a voltage-driven change of state, the steepness of the state function versus membrane potential curve defines the number of charges that move according to a Boltzmann distribution. On this basis, Hodgkin and Huxley (122–125) and Hodgkin, Huxley, and Katz (126) predicted that activation of sodium channels would require the movement of six positive charges from the intracellular to the extracellular side of the membrane. The movement of a larger number of charges through a proportionately smaller fraction of the membrane electrical field would be equivalent. Such a movement of membrane-bound charge gives rise to a capacitive current that can, in principle, be detected using electrophysiological techniques. Capacitive currents associated with activation of sodium channels (gating currents) have now been measured and analyzed in detail (13, 14). These gating currents represent charge movements associated with the change of sodium channel state from resting to active. Inactivation of sodium channels during a depolarizing prepulse blocks gating currents with the same time and voltage dependence as sodium currents. These experiments leave little doubt that the small capacitive currents measured are due to movements of charged groups on the sodium channel during activation. In all probability these charged groups are amino acids whose position in the protein structure is altered in the conformational change, which leads to activation. These results support a view of sodium channel activation as a major conformational change or sequence of conformational changes of the sodium channel protein that is driven by the force of the membrane electric field acting on protein-bound charges. This conformational change activates the transmembrane pore, which is spatially separate from the voltage sensors.

C. *Intracellular Components of Sodium Channels Involved in Inactivation*

Protein components located on the intracellular aspect of the sodium channel are essential for the inactivation of the sodium channel during a maintained depolarization. Intracellular perfusion of the squid giant axon with pronase, a mixture of proteolytic enzymes, blocks inactivation of the sodium channel in a time-dependent manner (15, 215). Alkaline protease b, the most substrate-specific enzyme of pronase, is responsible for the block of inactivation during intracellular perfusion with pronase (216). Trypsin also mimics the effect of alkaline protease b. Both these enzymes are specific for cleavage at the carboxyl group of lysyl and arginyl residues, suggesting that a protease-sensitive amino acid sequence containing lysine or arginine and located on the intracellular surface of the sodium channel is required for inactivation.

These results led Eaton et al. (82) to examine the effects of the arginine-specific reagents glyoxal, phenylglyoxal, and 2,3-butanedione on sodium channel inactivation. When perfused inside squid giant axons under voltage clamp, each of these reagents irreversibly blocked sodium channel inactivation. In addition, *N*-bromoacetamide and the tyrosine-specific reagents *N*-acetylimidazole (194) and tetranitromethane, or I^- plus lactoperoxidase (45), also block sodium channel inactivation when perfused inside the squid giant axon. A number of other amino acid-modifying reagents have no effect (194). Thus both arginine and tyrosine residues located on the intracellular aspect of the sodium channel are implicated in sodium channel inactivation. The action of both pronase and *N*-bromoacetamide to block inactivation is slightly voltage dependent, indicating that the susceptible residues are less available when the sodium channel is in the inactivated conformation (225).

III. NEUROTOXINS AS MOLECULAR PROBES OF SODIUM CHANNELS

Neurotoxins and drugs that alter sodium channel function affect one or more of the three essential functional properties of sodium channels: voltage-dependent activation, inactivation, or selective ion conductance. These toxins bind with high affinity and specificity to sodium channels and have been used as molecular probes to identify the protein components of sodium

TABLE 1. *Neurotoxin receptor sites on the sodium channel*

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

channels and to characterize functional regions within the sodium channel subunits.

Five groups of neurotoxins that act at different receptor sites on the sodium channel have been described (Table 1). Neurotoxin receptor *site 1* binds the water-soluble heterocyclic guanidines tetrodotoxin and saxitoxin. These toxins inhibit sodium channel ion transport by binding to a common receptor site that is thought to be located near the extracellular opening of the ion-conducting pore of the sodium channel (55, 120, 179, 210). In addition, recent work shows that polypeptide toxins from the marine snail *Conus geographus* also inhibit sodium channels by binding at the same receptor site (173, 191, 284). Multiple arginine residues in these polypeptide toxins may serve to bind to the same subsites as the guanidinium moieties of tetrodotoxin and saxitoxin.

Neurotoxin receptor *site 2* binds several lipid-soluble toxins, including grayanotoxin and the alkaloids veratridine, aconitine, and batrachotoxin (5, 55, 121, 138, 179, 267). Competitive interactions of these four toxins at neurotoxin receptor *site 2* have been demonstrated by ion flux studies and by direct measurements of specific binding of [^{3}H]batrachotoxinin A 20α-benzoate to sodium channels (55, 62). These toxins cause persistent activation of sodium channels at the resting membrane potential by blocking sodium channel inactivation and shifting the voltage dependence of channel activation to more negative membrane potentials. These effects result from preferential binding of the toxins with high affinity to the active states of sodium channels and consequent stabilization of those states, as described by an allosteric model of neurotoxin binding and action (53, 55). The toxins acting at neurotoxin receptor *site 2* also alter the ion selectivity of the sodium channel (55, 121, 138). Therefore neurotoxin receptor *site 2* is likely to be localized on a region of the sodium channel that is involved in voltage-dependent activation and inactivation and is allosterically linked to the transmembrane pore of the channel.

Neurotoxin receptor *site 3* binds polypeptide toxins purified from North African scorpion venoms or sea anemone nematocysts (55, 252). These toxins slow or block sodium channel inactivation (36, 55, 146, 179, 252). They also enhance persistent activation of sodium channels by the lipid-soluble toxins acting at neurotoxin receptor *site 2* (55, 146). The affinity for binding of ^{125}I-labeled derivatives of the polypeptide toxins to neurotoxin receptor *site 3* is reduced by depolarization, and the voltage dependence of scorpion toxin binding is correlated with the voltage dependence of sodium channel activation (52, 54, 55). These data indicate that neurotoxin receptor *site 3* is located on a region of the sodium channel that undergoes a conformational change during voltage-dependent channel activation, leading to reduced affinity for scorpion toxin. These results provided a second independent line of evidence, in addition to the measurements of activation gating currents, supporting the concept of voltage-dependent conformational change as an important component of the mechanism of sodium channel activation. Thus scorpion toxin and sea anemone toxin are likely to bind to voltage-sensing or gating structures involved in activation of sodium channels and to slow or block the normal coupling of the activation of the channel to inactivation.

Neurotoxin receptor *site 4* binds a second class of scorpion toxins that are homologous to those that bind at receptor *site 3* (74, 146, 252). These toxins, which are most prominent in the venoms of New World scorpions like *Centruroides sculpturatus* and *Tityus serrulatus*, shift the voltage dependence of sodium channel activation to more negative membrane potentials without modifying sodium channel inactivation (47, 74, 170). They do not alter the binding of neurotoxins at *site 3*, and their binding to sodium channels is unaffected by toxins that bind at neurotoxin receptor *site 3*. These results indicate that this second class of scorpion toxins binds to a fourth receptor site on the sodium channel. They have been designated β-scorpion toxins to distinguish them from the α-scorpion toxins that bind at *site 3* and slow sodium channel inactivation.

Neurotoxin receptor *site 5* binds two classes of novel, lipid-soluble polyether toxins with molecular masses in the range of 1,000 Da, the brevetoxins and the ciguatoxins (61, 64, 156, 197, 238). The brevetoxins are formed from 11 fused six- and seven-membered ether rings arranged in a crescent shape (154). These unusual molecules cause repetitive firing of nerves, shift the voltage dependence of sodium channel activation, and block inactivation (34, 127). These actions resemble those of neurotoxins, which act at receptor *site 2*. However, brevetoxins have no effect on neurotoxin binding at receptor *sites 1* and *3* and allosterically enhance the binding and action of neurotoxins that act at neurotoxin receptor *sites 2* and *4* (64, 238). These results show that they exert their effects on sodium channel activation and inactivation at a fifth receptor site on the sodium channel. This site has been detected in direct binding studies using a radiolabeled derivative of brevetoxin (197), and the ciguatoxins have been shown to inhibit brevetoxin binding (156).

Each of the neurotoxins described has been shown to act on one of the five specific neurotoxin receptor sites on sodium channels by direct measurement of spe-

cific binding of radiolabeled toxin or by measurement of competitive displacement of the binding of other radiolabeled neurotoxins. Additional classes of toxins have well-defined effects on sodium channel function but have not been found to interact with any of these five sites. These toxins are likely to act at one or more receptor sites on the sodium channel that do not overlap with neurotoxin receptor *sites 1–5*. Thus the sodium channel is the molecular target for a surprisingly rich array of neurotoxins. In addition to providing a varied arsenal for defense of venomous animals and for attack on their prey, these many different toxins also provide a versatile set of molecular tools for studies of the structure and function of sodium channels.

IV. PURIFICATION AND RECONSTITUTION OF SODIUM CHANNEL FUNCTION

A. Sodium Channel Subunits in the Membrane

1. Covalent labeling of polypeptide components

Direct chemical identification of sodium channel components in situ was first achieved by specific covalent labeling of neurotoxin receptor *site 3* with a photoreactive azidonitrobenzoyl derivative of the scorpion toxin from the North African scorpion *Leiurus quinquestriatus* (32). Irradiation with ultraviolet light causes covalent attachment of the specifically bound toxin derivative. Analysis of covalently labeled synaptosomes by polyacrylamide gel electrophoresis under denaturing conditions in sodium dodecyl sulfate revealed specific covalent labeling of two polypeptides of 260 and 36 kDa that have subsequently been designated the α- and β1-subunits of the sodium channel. The covalent labeling of these two polypeptides in synaptosomes was shown to be specific by inhibition with unlabeled scorpion toxin or by block of voltage-dependent binding of scorpion toxin by membrane depolarization. The β-scorpion toxins derived from American scorpion venoms have been used to label neurotoxin receptor *site 4* on the sodium channel (25, 76, 134). Toxin-γ from *T. serrulatus* was attached to its receptor site by covalent cross-linking with disuccinimidyl suberate. A single polypeptide of 270 kDa was labeled in rat brain synaptosomes. In contrast, photoreactive derivatives of toxin II from *Centruroides suffusus suffusus* label two polypeptides with molecular masses similar to the α- and β1-subunits of the channel. These results suggest that neurotoxin receptor *site 4*, like *site 3*, is located near the contact regions of the α- and β1-subunits of the sodium channel.

All of these labeling methods result in covalent attachment of amino groups in the toxins to nearby components of the sodium channel. Because each toxin contains multiple amino groups, the differential labeling of the α- and β1-subunits of the sodium channel that has been observed may result from reaction with amino groups in different regions of the toxin. Evidence in favor of this has been developed by separation of photoreactive derivatives of the α-scorpion toxin from *L. quinquestriatus* by ion-exchange chromatography (237). One derivative labeled only the α-subunit, whereas the other preferentially labeled the β1-subunit. Evidently, individual amino groups on the *Leiurus* toxin can be in proximity to either the α- or β1-subunit when the toxin is bound at neurotoxin receptor *site 3*.

2. Radiation inactivation

The mass of functional units of membrane proteins can be estimated by measuring the target size for inactivation of that function by irradiation with high-energy X rays. Targets with larger sizes are inactivated more rapidly. A single hit anywhere within the covalently bonded structure of the target protein is considered to be sufficient to inactivate the entire molecule. Measurements of the target size for inactivation of tetrodotoxin binding to the sodium channel in electroplax or brain membranes show that a structure of 230–260 kDa is required for this activity (25, 151). This mass corresponds approximately to that of the α-subunit, providing early evidence that this subunit is required for saxitoxin and tetrodotoxin binding.

More recent studies of radiation inactivation of scorpion toxin binding activity implicate both α- and β-subunits. A target size of 263 kDa was observed for binding of α-scorpion toxins (25). Target sizes of 266 and 45 kDa were observed for binding of β-scorpion toxins, suggesting a requirement for both α- and β1- or β2-subunits for high-affinity β-scorpion toxin binding (10, 25).

B. Isolation of Sodium Channels

1. Solubilization and purification

An alternative approach to identification and characterization of the protein components of the sodium channel is to solubilize neurotoxin binding activity with detergents and to purify the solubilized sodium channel components using neurotoxin binding as a specific assay. Henderson and Wang (115) and Benzer and Raftery (35) first showed that the tetrodotoxin binding component of sodium channels in garfish olfactory nerve could be solubilized by nonionic detergents with retention of high affinity and specificity of toxin binding. Subsequent work extended these findings to sodium channels in eel electroplax (2), mammalian brain (63, 144), mammalian skeletal muscle (23), and chicken and mammalian heart (79, 158). In contrast to the ease of solubilization of neurotoxin receptor *site 1* with retention of saxitoxin and tetrodotoxin binding activity, both neurotoxin receptor *site 2* and neurotoxin receptor *site 3* (62, 63) lose high-affinity neurotoxin binding activity on solubilization. Binding activity for some neurotoxins at neuro-

toxin receptor *site 4* is retained after solubilization (158, 185).

Although the sodium channel was successfully solubilized in 1972, the marked instability of the toxin binding activity prevented progress on the purification of the sodium channel until the discovery that addition of phospholipid or phospholipid and calcium to the detergent-solubilized sodium channel markedly stabilized the saxitoxin binding activity (2, 4, 63). This stabilization is proposed to result from formation of mixed detergent-phospholipid micelles having energetically favorable interaction with the solubilized channel. The broad specificity of this phospholipid requirement is consistent with this interpretation.

The sodium channels from electric eel electroplax (3, 172, 185), rat brain (108, 109, 112), rat and rabbit skeletal muscle (21, 142), and chicken heart (158) were initially purified to near homogeneity using a combination of conventional purification methods: anion-exchange chromatography, adsorption chromatography on hydroxylapatite, affinity chromatography on wheat germ agglutinin-Sepharose, and size fractionation by velocity sedimentation in sucrose gradients or by gel filtration chromatography. Preparations from each of these tissues made by conventional procedures bind up to 0.9 mol [^{3}H]saxitoxin or [^{3}H]tetrodotoxin per mole of protein, indicating a high degree of purity. More recently, immunoaffinity purification using monoclonal antibodies against the α-subunit protein has allowed rapid isolation of denatured sodium channels from eel electroplax or denatured α-subunits from skeletal muscle (50, 178). Affinity chromatography on immobilized immunoglobulin (Ig) M directed against α-(2,8)-sialic acid or on *Limax flavus* lectin, which also binds poly-(2,8)-sialic acid, has been shown to give a one-step purification of the sodium channel from eel electroplax to a specific binding capacity of ~1 mol [^{3}H]tetrodotoxin/mol (132). These solubilized and purified channel preparations provided the necessary material for analysis of the molecular properties of the sodium channel.

2. *Molecular mass of solubilized sodium channel*

The mass of the solubilized sodium channel was estimated by hydrodynamic studies. The solubilized sodium channel-Triton X-100 complex from rat brain has a Stokes radius of 80 Å, a sedimentation coefficient of 12 S, and a partial specific volume of 0.82 cm^3/g (110). These data define a molecular mass of 601 kDa for the protein-detergent complex. The contribution of protein and bound detergent was estimated by comparison of the partial specific volume of the complex (0.82 cm^3/g), with values for Triton X-100-phosphatidylcholine (5:1) (0.92 cm^3/g) and for typical proteins (0.73 cm^3/g). This comparison indicated that the protein-detergent complex contains 0.9 g Triton X-100-phosphatidylcholine/g protein. The molecular mass of the sodium channel protein is therefore 316 kDa. A similar analysis of the sodium channel from skeletal muscle solubilized in Lubrol PX arrived at an estimate of 314 kDa (24).

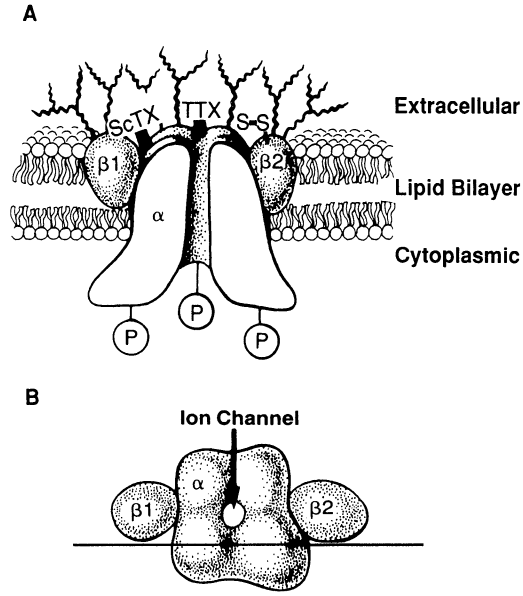


FIG. 2. Subunit structure of brain sodium channel. *A*: view of a cross section of a hypothetical sodium channel consisting of a single transmembrane α-subunit of 260 kDa in association with a β1-subunit of 36 kDa and a β2-subunit of 33 kDa. β1-Subunit is associated noncovalently, whereas β2-subunit is linked through disulfide bonds. All 3 subunits are heavily glycosylated on their extracellular surfaces, and α-subunit has receptor sites for α-scorpion toxins (ScTx) and tetrodotoxin (TTX). Intracellular surface of α-subunit is phosphorylated by multiple protein kinases (P). *B*: view of sodium channel from extracellular side illustrating formation of transmembrane pore in center of α-subunit.

3. *Subunit composition of purified sodium channel*

The initial identification of the subunits of the sodium channel from rat brain by photoaffinity labeling in situ revealed two classes of polypeptides of 260 and 36 kDa (32). Analysis of the purified sodium channel from rat brain has shown that it consists of three polypeptides (Fig. 2): α of 260 kDa, β1 of 36 kDa, and β2 of 33 kDa (108, 109, 112, 167). All three subunits are intrinsic membrane glycoproteins. The α- and β1-subunits, but not the β2-subunit, are covalently labeled by photoreactive scorpion toxin derivatives (32, 237). The β2-subunit is covalently attached to the α-subunit by disulfide bonds, whereas the β1-subunit is associated noncovalently (112, 167). The subunits appear to be present in a 1:1:1 stoichiometry (109), and the sum of their molecular masses (329 kDa) agrees closely with the oligomeric molecular mass of the solubilized sodium channel (316 kDa; Ref. 110). Antibodies against either the β1- or β2-subunits immunoprecipitate nearly all brain sodium channels, indicating that most, if not all, have a heterotrimeric structure (165, 282).

The sodium channel purified from rat skeletal muscle sarcolemma initially appeared to have a different subunit composition. However, subsequent work with highly purified preparations made under conditions that limit proteolysis showed that both sarcolemmal and transverse tubule sodium channels consist of a ma-

jor subunit of 260 kDa and one or two smaller subunits of 38 kDa (21, 142).

Sodium channel preparations from eel electroplax (3, 132, 172, 185) and chicken heart (158) contain only a single polypeptide of 260 kDa. This polypeptide is analogous in mass and amino acid sequence to the α-subunit of the sodium channel from brain and skeletal muscle (see sect. v*A*).

C. *Reconstitution of Sodium Channel Function From Purified Components*

Restoration of full sodium channel function by incorporation of purified components into phospholipid vesicles provided rigorous proof that the proteins identified and purified on the basis of their neurotoxin binding activity are indeed sufficient to form a functional voltage-gated sodium channel. The goal of the reconstitution experiments was to show, as quantitatively as possible, that purified sodium channel preparations of known protein composition can mediate normal sodium channel functions. In addition, successful reconstitution provides a valuable experimental preparation for biochemical analysis of the structure and function of sodium channels.

1. *Ion flux*

Purified sodium channels from rat skeletal muscle, rat brain, and eel electroplax have been incorporated into phosphatidylcholine vesicles by mixture with the phospholipid dispersed in nonionic detergent, followed by slow removal of detergent by adsorption to polystyrene beads (217, 257, 273). Single-walled phosphatidylcholine vesicles containing sodium channels are formed by this method. The channels are oriented approximately randomly with one-half of the saxitoxin/tetrodotoxin binding sites directed outward as in intact cells and one-half directed inward in inside-out configuration. Activation of the reconstituted sodium channels by incubation with veratridine or batrachotoxin increases the initial rate of $^{22}Na^+$ influx into the reconstituted channels 4- to 15-fold. Outside-out and inside-out channels contribute to the measured $^{22}Na^+$ influx equivalently. Appropriate concentrations of the alkaloid toxins are effective in activating the purified channels, and they are inhibited by appropriate concentrations of tetrodotoxin and local anesthetics. The measured ion selectivity is $Na^+ > K^+ > Rb^+ > Cs^+$, as for native sodium channels activated by alkaloid neurotoxins (142, 259, 260). The toxin-modified sodium channels in reconstituted phospholipid vesicles retain responsiveness to membrane potential and can be activated by depolarization of the vesicles using changes in ionic gradients to control the membrane potential in the vesicles (90). Thus there is strong evidence that at least a fraction of the sodium channel molecules in highly purified preparations retain a selective ion channel that can be activated and inhibited by neurotoxins and drugs.

It is important in reconstitution experiments to estimate the fraction of purified sodium channel protein molecules whose ion transport function can be restored. Only if a large fraction of the purified protein molecules is active in ion transport can it be concluded that the major species characterized biochemically is functionally active. Such estimates have been made by comparing the initial rate of neurotoxin-stimulated $^{22}Na^+$ influx in reconstituted vesicles to that in intact cells and by preparing vesicles having a mean of less than one reconstituted sodium channel each (4–6 pmol sodium channel/mg lipid), estimating the fraction of vesicles whose internal volume is accessible to neurotoxin-activated sodium channels and applying appropriate statistical procedures to calculate the fraction of functionally active channels. Both these approaches lead to the conclusion that at least 30% and perhaps as many as 70% of the purified channels from rat brain and eel electroplax are functionally active (81, 259). Because the protein preparations from rat brain were judged 90% pure and no single contaminant comprised >2%, it was concluded that a purified complex of α-, β1-, and β2-subunits was sufficient to mediate neurotoxin-activated ion flux (109, 259). Similar experiments on sodium channels purified from eel electroplax showed that a large fraction of those purified channels are active in ion conductance as well, even though they contain only an α-subunit (81). Evidently, the α-subunit of the purified sodium channel alone can function as an ion channel when activated by neurotoxins in purified preparations. Although purified electroplax sodium channels have only α-subunits, dissociation of β1-subunits from the α-subunits of rat brain sodium channels under mild conditions causes loss of functional activity (168, 169). In contrast, dissociation of β2-subunits does not affect the function of brain sodium channels.

Nearly homogeneous sodium channel preparations from rat skeletal muscle reconstituted at ~5 pmol/mg phospholipid yield half times for vesicle $^{22}Na^+$ equilibration of <50 ms in the absence of ion gradients or a membrane potential (142). Similarly, purified preparations of sodium channels from eel electroplax studied using a fluorescence assay for neurotoxin-activated ion flux in reconstituted vesicles gave very rapid rates of sodium flux comparable to single-channel currents measured physiologically (265). These rapid rates achieved with a low density of sodium channels in the vesicle bilayer also support the conclusion that a substantial fraction of the purified skeletal muscle and eel electroplax channels are functionally active.

2. *Voltage-dependent neurotoxin binding*

Although reconstitution into phosphatidylcholine vesicles is sufficient to restore neurotoxin-stimulated ion transport activity of the purified brain sodium channel, high-affinity binding of the α-scorpion toxin

from *L. quinquestriatus* is not restored under these conditions (259). Because binding of toxins at neurotoxin receptor *site 3* is voltage and state dependent (for review see Ref. 55), the failure to recover this binding activity likely indicates that the channel is locked in a state with low affinity for scorpion toxin. Reconstitution in mixtures of phosphatidylcholine and mixed brain lipids restores scorpion toxin binding (259). The requirement for brain lipids can be satisfied by addition of brain phosphatidylethanolamine alone or in combination with phosphatidylserine (86). Other minor brain lipids did not have detectable effects. In vesicles of 65% phosphatidylcholine-35% phosphatidylethanolamine, high-affinity binding of *Leiurus* scorpion toxin is restored in good yield (50–75%) and is voltage dependent. Evidently, the native voltage-dependent functional properties of the sodium channel from rat brain require reconstitution in an appropriate phospholipid environment.

3. *Single-channel recording*

The ion conductance of the sodium channel is normally regulated by changes in membrane potential on the millisecond time scale. This aspect of function of purified and reconstituted sodium channels has been studied by two different single-channel recording methods. These techniques have excellent time resolution and voltage control and are sufficiently sensitive to detect the square pulses of ionic conductance mediated by a single purified sodium channel. They have the disadvantage that the fraction of purified sodium channels whose functional activity has been restored cannot be determined.

Rosenberg et al. (218) used a freeze-thaw method to prepare large (5–20 μm) reconstituted vesicles of mixed phospholipid composition (phosphatidylethanolamine-phosphatidylserine-phosphatidylcholine = 5:4:1) containing purified electroplax sodium channels and recorded from excised patches of membrane attached across the tip of the micropipette. In some patches, single-channel currents were observed that had the appropriate conductance (11 pS), ion selectivity [Na-to-K permeability ratio (P_{Na}/P_K) = 7], and time and voltage dependence of opening and closing to represent the activity of individual sodium channels. However, the number of active single channels detected was very small and not easily reproducible among different purified sodium channel preparations.

An alternative approach is to incorporate reconstituted sodium channels into planar phospholipid bilayers. The high electrical resistance of such bilayers allows measurement of the ion conductance mediated by a single ion channel. After treatment with batrachotoxin, sodium channels in synaptosomal membrane vesicles can be successfully incorporated into planar phospholipid bilayers by fusion of the membrane vesicles with preformed bilayers (145). The functional properties of batrachotoxin-modified sodium channels in native membranes are retained. The presence of batrachotoxin is required to block sodium channel inactivation and allows measurement of steady-state opening and closing of sodium channels as a function of time and membrane voltage.

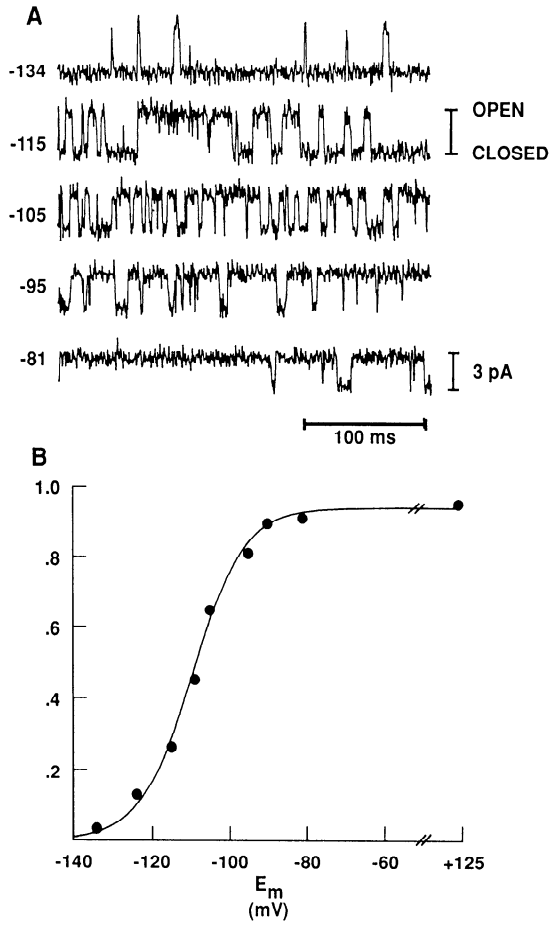


FIG. 3. Voltage-dependent opening of purified and reconstituted sodium channels. Purified brain sodium channels containing only α- and β1-subunits were reconstituted into phospholipid vesicles composed of phosphatidylethanolamine and phosphatidylcholine and activated by incubation with batrachotoxin. Reconstituted vesicles were fused to planar phospholipid bilayers for single-channel recording. *A*: channel activity was recorded at indicated membrane potentials. *B*: percent of time that single channel in *A* was in open state was calculated and plotted against membrane potential (E_m).

This recording technique has been applied to purified and reconstituted sodium channels from rat brain that consisted of a stoichiometric complex of α-, β1-, and β2-subunits (111), purified skeletal muscle sodium channels consisting of α- and β1-like subunits (90), and purified eel electroplax sodium channels consisting only of α-like subunits (205). For channels purified from rat brain, fusion of reconstituted vesicles in the presence of batrachotoxin was accompanied by the appearance of single-channel conductance events of 25 pS (Fig. 3). The probability of channel opening was voltage dependent, increasing from near 0 at −120 mV to 96.8% at all voltages more positive than −60 mV (Fig. 3). The channels were open one-half of the time at −91 mV, and the steepness of their voltage dependence corresponded to an apparent gating charge of 3.8 mV. Their ion selectiv-

ity ($P_{Na}/P_K/P_{Rb}$ = 1:0.13:0.04), block by tetrodotoxin [dissociation constant (K_D) = 8.3 nM at −50 mV, 135 nM at 70 mV], single-channel conductance, and voltage dependence all agree precisely with those of native sodium channels in the presence of batrachotoxin. Generally similar results were obtained for skeletal muscle and eel electroplax sodium channels. The results show that the voltage-dependent gating function, high single-channel conductance, and ion selectivity of the purified sodium channels recorded in these planar bilayers remain quantitatively intact after purification and reconstitution.

Activation of sodium channels by alkaloid neurotoxins causes a number of changes in their properties, including block of channel inactivation, shift of the voltage dependence of activation, and change in ion permeability properties. Thus it would be valuable to be able to study purified and reconstituted sodium channels by patch-clamp methods without toxin activation. Correa et al. (70) used highly purified eel electroplax sodium channels prepared in one step by affinity chromatography on the α-(2,8)-sialic acid-specific *L. flavus* agglutinin (132) and an improved version of the reconstitution method of Rosenberg et al. (218) to study the functional properties of purified sodium channels without toxin activation. They recorded single sodium channel currents of up to 30 pS that were sodium selective but much less strongly so than native sodium channels. Unusually strong depolarization (more positive than −10 mV) was required to activate the purified and reconstituted sodium channels, and the overall voltage dependence of gating was shifted 50 mV toward more positive membrane potentials. Two single-channel gating patterns were recorded. In one pattern, individual channels opened soon after depolarization for brief periods and then closed and inactivated, a normal pattern of gating for sodium channels. In the second more common pattern, sodium channels opened for prolonged periods and reopened repeatedly during long depolarizations. Evidently, the inactivation gating properties of these purified and reconstituted sodium channels is altered from normal. Even though the functional properties of native sodium channels are not faithfully reproduced in this reconstitution system, the results are important because they show that purified and reconstituted sodium channels that have not been toxin activated do retain the basic aspects of sodium channel function.

Considered together, the results of the different reconstitution studies complement each other and establish that the purified sodium channels from various sources retain their functional properties. The ion flux results for rat brain, skeletal muscle, and eel electroplax show that the isolated sodium channel complexes of one to three nonidentical glycoprotein subunits are sufficient to form a selective, neurotoxin-activated ion channel. Patch-clamp recordings from reconstituted electroplax sodium channels show that some of these isolated proteins can be activated by depolarization and retain a high single-channel conductance with sodium-specific ion selectivity in the absence of neurotoxins. Planar bilayer recordings of reconstituted sodium channels in the presence of batrachotoxin show that the voltage dependence of channel gating and the rate and selectivity of ion transport are quantitatively retained for the toxin-modified sodium channels.

V. STRUCTURE AND FUNCTION

A. *Primary Structures of Sodium Channel α-Subunits*

Availability of purified and functionally characterized sodium channel preparations provided the necessary starting material for identification of the genes encoding the sodium channel subunits and determination of their primary structures. Using oligonucleotides encoding short segments of the electric eel electroplax sodium channel and antibodies directed against it, Noda et al. (182) isolated cDNAs encoding the entire polypeptide from expression libraries of electroplax mRNA. The deduced amino acid sequence revealed a protein with four internally homologous domains, each containing multiple potential α-helical transmembrane segments (Fig. 4). The wealth of information contained in this deduced primary structure has revolutionized research on sodium channels.

The cDNAs encoding the electroplax sodium channel were used to isolate cDNAs encoding three distinct, but highly homologous, rat brain sodium channels (types I, II, and III; see Refs. 137, 180). The cDNAs encoding the alternatively spliced type IIA sodium channel were isolated independently by screening expression libraries with antibodies against the rat brain sodium channel α-subunit (17, 95). The type II gene contains two adjacent exons encoding segment IS3 (227) that are alternatively spliced into mature mRNA in a developmentally regulated manner. The type II form is most prominent in embryonic and neonatal brain, whereas the type IIA form is most prominent in the adult brain (227, 287). The cDNAs encoding the type II/IIA sodium channel were used as probes to isolate cDNAs encoding sodium channel α-subunits expressed in skeletal muscle and heart by low-stringency hybridization (135, 213, 266). The μ1-sodium channel α-subunit is expressed primarily in adult skeletal muscle (266); the h1 sodium channel α-subunit is expressed primarily in heart and also in uninnervated or denervated skeletal muscle (135, 213). These sodium channels have a close structural relationship to the three brain sodium channel α-subunits. In general, the similarity in amino acid sequence is greatest in the homologous domains from transmembrane segment S1–S6, whereas the intracellular connecting loops are not highly conserved.

The cDNAs encoding two distinct sodium channels from *Drosophila* have been cloned by cross-hybridization, and parts of the primary structures of the corresponding sodium channels have been deduced (203, 226). Although these cDNAs encode proteins related to mammalian sodium channels, their function as sodium channels has not been demonstrated in *Drosophila*. In addi-

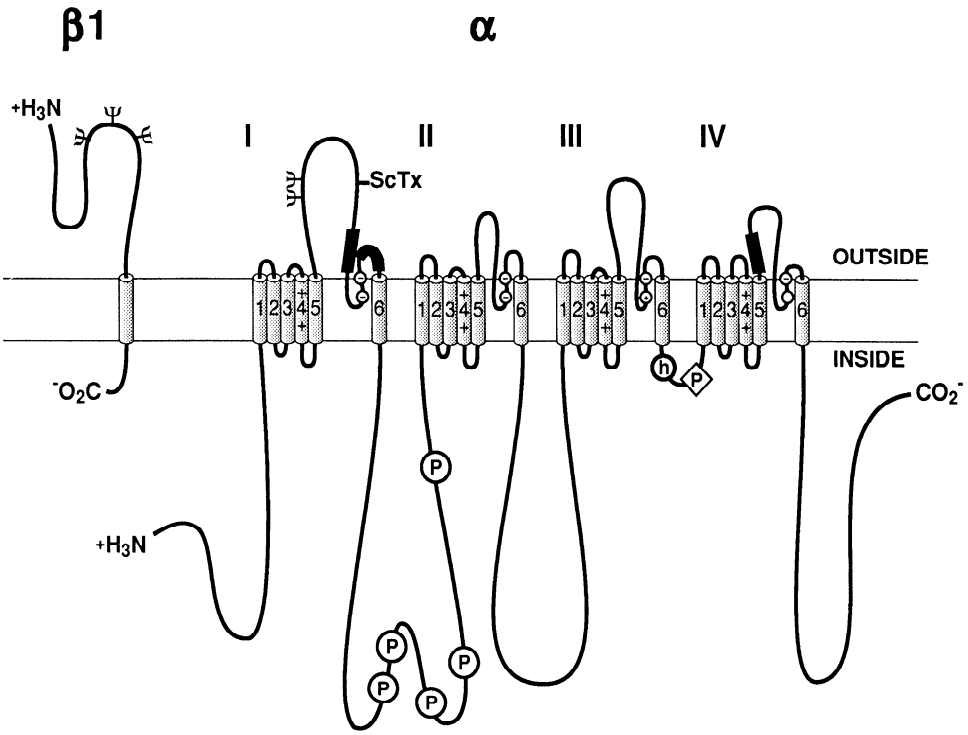


FIG. 4. Primary structures of α- and β1-subunits of sodium channel illustrated as transmembrane folding diagrams. Bold line, polypeptide chains of α- and β1-subunits with length of each segment approximately proportional to its true length in rat brain sodium channel. Cylinders represent probable transmembrane α-helices. Other probable membrane associated segments are drawn as loops in extended conformation like remainder of sequence. Sites of experimentally demonstrated glycosylation (ψ), cAMP-dependent phosphorylation (P in a circle), protein kinase C phosphorylation (P in a diamond), amino acid residues required for tetrodotoxin binding (small circles with +, −, or open fields depict positively charged [Lys^{1422}], negatively charged, or neutral [Ala^{1714}] residues, respectively), and amino acid residues that form inactivation particle (h in a circle).

tion, the genomic DNA from the *para* locus, which specifies a paralyzed phenotype, has been cloned, and the exons encoding a *Drosophila* sodium channel have been completely sequenced (160). The *para* sodium channel is remarkably closely related to the rat brain sodium channels. The close sequence similarity to rat brain sodium channels and the physiological and pharmacological properties of the mutations at this locus that cause the *para* phenotype indicate that the *para* gene product is indeed a functional sodium channel in *Drosophila*. Thus it appears that *Drosophila* also has multiple sodium channel genes as observed in rats. Presumably these distinct genes have distinct roles in electrical excitability in both species.

Most recently, a new putative sodium channel α-subunit has been cloned from glial (92) and heart (93) cDNA libraries. Its amino acid sequence retains the four-domain structure of the other α-subunits and many of their other conserved features, but it is distinctly more divergent than the other α-subunits previously characterized. It has been suggested that this glial/heart sodium channel is the first member of a new subfamily of sodium channels (93). It is strongly expressed in the heart and uterine smooth muscle (93). It appears to be responsible for the sodium currents recorded in astrocytes in cell culture and is expressed in other glia that generate significant sodium currents (92).

B. Primary Structures of Sodium Channel β-Subunits

The primary structures of sodium channel β1-subunits have been determined only recently (129). The β1-subunit cloned from rat brain is a small protein of 218 amino acids (22,821 Da), with a substantial extracellular domain having four potential sites of *N*-linked glycosylation, a single α-helical membrane-spanning segment, and a very small intracellular domain. Experiments with anti-β1-subunit antibodies (165) indicate that there is a small family of β1-subunits. These distinct β1-subunits may form specific associations with different α-subunits and contribute to the diversity of sodium channel structure and function. The β2-subunits have not yet been cloned, but it may be anticipated that they may also add to the potential diversity of sodium channel structure and function.

C. Functional Expression of Sodium Channel Subunits

The α-subunit mRNAs isolated from rat brain by specific hybrid selection with type IIA cDNAs (95) and the RNAs transcribed from cloned cDNAs encoding α-subunits of rat brain (17, 174, 181, 256), skeletal muscle (266), or heart (75, 280) sodium channels are sufficient to direct the synthesis of functional sodium channels when injected into *Xenopus* oocytes. These results establish that the protein structures necessary for voltage-dependent gating and ion conductance are contained within the α-subunit itself, as suggested from the work on purified and reconstituted sodium channels.

Although α-subunits alone are sufficient to encode functional sodium channels, their properties are not normal. Inactivation is slow relative to that observed in intact neurons or muscle cells, and its voltage dependence is shifted to more positive membrane potentials. Coexpression of low-molecular-mass RNA from brain or skeletal muscle can accelerate inactivation, shift its voltage dependence to more negative membrane poten-

tials, and increase the level of expressed sodium current (17, 141, 266). These results suggested that the low-molecular-mass β1- or β2-subunits may modulate functional expression of the α-subunit. Coexpression of RNA transcribed from cloned β1-subunits directly demonstrates this modulation (129). Coexpression of β1-subunits in *Xenopus* oocytes accelerates the decay of the sodium current 5-fold, shifts the voltage dependence of sodium channel inactivation 20 mV in the negative direction, and increases the level of sodium current 2.5-fold. Evidently, β1-subunits are essential for normal functional expression of rat brain sodium channels.

Sodium channel α-subunits can also be functionally expressed in mammalian cells in culture. Stable lines of Chinese hamster ovary (CHO) cells expressing the type IIA sodium channel generate sodium currents with a normal time course and voltage dependence, even though there is no evidence that these cells express an endogenous β1-subunit to form a complex with the transfected α-subunit (231, 277). Evidently, β1-subunits do not have as important a functional impact when the α-subunit is expressed in the genetic background of a mammalian somatic cell. The α-subunits expressed in CHO cells have normal pharmacological properties as well. They have high-affinity receptor sites for saxitoxin and tetrodotoxin and are inhibited by low concentrations of tetrodotoxin. The voltage dependence of their activation is shifted in the negative direction, and they are persistently activated by veratridine in a stimulus-dependent manner. Their inactivation is slowed by α-scorpion toxins. In addition, they are inhibited in a strongly frequency- and voltage-dependent manner by local anesthetic, antiarrhythmic, and anticonvulsant drugs (202). Thus the receptor sites for all of these diverse pharmacological agents are located on the α-subunits.

D. Functional Map of Sodium Channel α-Subunits

A major goal of current research on the voltage-gated ion channels is to define the structural components that are responsible for specific aspects of channel function. Two main experimental approaches have proven valuable in these studies. Antibodies against short, ~20-residue peptide segments of the principal α-subunits of the sodium channels have been used to probe domains that are required for specific channel functions or that can be covalently labeled by neurotoxins or protein phosphorylation. Mutations have been introduced into cDNAs encoding the principal α-subunits by oligonucleotide-directed mutagenesis, expressed in recipient cells, and analyzed by electrophysiological recording.

1. Voltage-dependent activation

The steep voltage dependence of activation of the voltage-sensitive ion channels is their most unique characteristic. It requires that they have charged amino acid residues or strongly oriented dipoles within the membrane electric field of the phospholipid bilayer. For example, Hodgkin and Huxley (122–125) and Hodgkin, Huxley, and Katz (126) pointed out in their classic 1952 papers that the steepness of voltage-dependent activation of sodium channels requires the movement of the equivalent of six protein-bound positive charges from the inner surface of the bilayer membrane to the outer surface during activation or the movement of a larger number of charges a proportionally smaller distance across the membrane. The movement of these gating charges or voltage sensors under the force of the electric field is believed to initiate a conformational change in the channel protein resulting in activation. Because activation of the sodium channel is rapid, the movement of its gating charges across the membrane causes a measurable capacitive gating current that has been detected in voltage-clamp experiments (13, 14). This movement of gating charge begins immediately on depolarization of the membrane, is largely complete before movement of ionic current through the open channel is detected, and is blocked if the sodium channel is first inactivated before depolarization.

The requirement for transmembrane movement of multiple charges during sodium channel activation has focused attention on the S4 segments of the voltage-sensitive ion channels that are both positively charged and hydrophobic. These unique structures, which consist of repeated motifs of a positively charged amino acid residue, usually arginine, followed by two hydrophobic residues (Fig. 5), were first observed in the amino acid sequence of the electroplax sodium channel (182). Conservation of this amino acid sequence among different voltage-sensitive ion channels, first noted for sodium channels from electroplax and brain, is striking across this broad range of ion channels from diverse species.

Several authors have independently proposed that these S4 segments have a transmembrane orientation and are the gating charges or voltage sensors of the sodium channel (57, 58, 101, 103, 104, 180, 181). Figure 5 illustrates the "sliding helix" model of voltage-dependent gating (58). The S4 segments are proposed to adopt an α-helical conformation. In this conformation, the arginine residues form a spiral ribbon of positive charge around the core of the helix. They are stabilized in their transmembrane position by formation of ion pairs between the positive charges of the S4 segments and negatively charged amino acid residues from the surrounding transmembrane α-helices of the S1, S2, and S3 segments. This arrangement forms a spiral staircase of ion pairs across the membrane (Fig. 5). This ion pairing arrangement is metastable, held in place by the force of the electric field drawing the positive charges into the cell and repelling the negative charges outward at a typical resting membrane potential of −80 mV. On depolarization, this force is relieved and the S4 segment is released to slide outward along a spiral path and form a new set of ion pairs. This sliding motion of the S4 helix transfers the equivalent of a full positive charge across

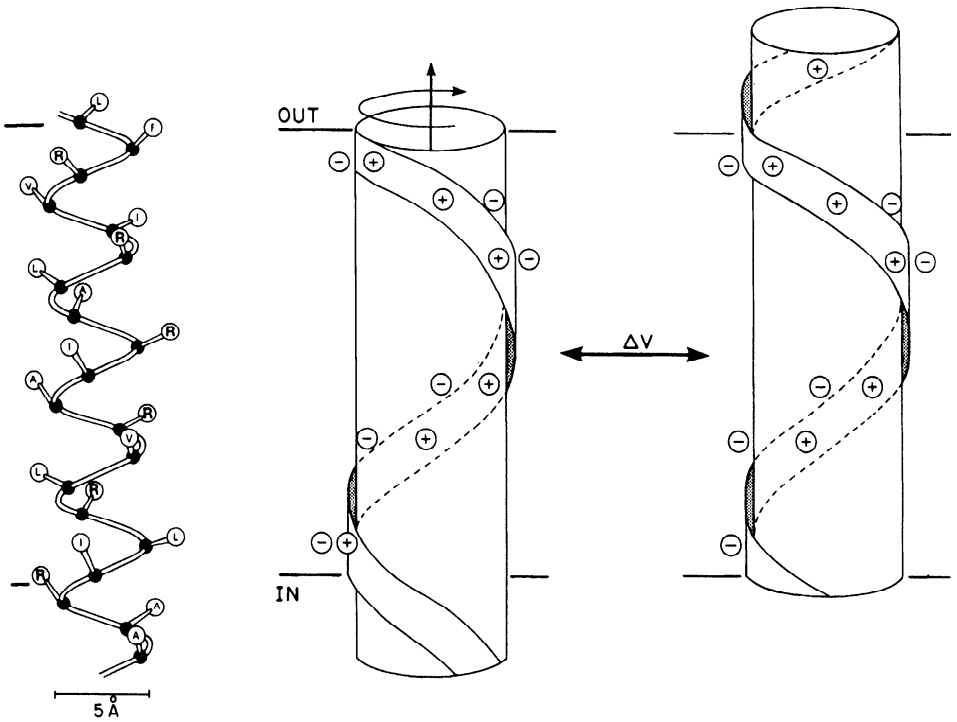


FIG. 5. Sliding helix model of voltage-dependent activation. *Left*: ball-and-stick, 3-dimensional representation of S4 helix of domain IV of sodium channel. Darkened circles represent α-carbon of each amino acid residue. Open circles specify amino acids in single letter code and show direction of projection of side chain away from core of helix. Positively charged amino acids are indicated in bold letters. *Right*: movement of S4 helix in response to depolarization [change in voltage (ΔV)]. Transmembrane helix S4 is represented as a cylinder with a spiral ribbon of positive charge. At resting membrane potential (*left*), all positively charged amino acid resides are paired with fixed negative charges on other transmembrane segments of channel, and S4 segment is maintained in that position by force of negative internal membrane potential. Depolarization reduces force holding positive charges in their inward position. S4 helix is then proposed to undergo a spiral motion through a rotation of ~60° and outward displacement of ~5 Å. This movement leaves an unpaired negative charge on inner surface of membrane and reveals an unpaired positive charge on external surface of membrane to give a net gating charge transfer (ΔQ) of +1.

the membrane with only a 5 Å outward movement and a 60° rotation. Sequential, voltage-driven movements of the S4 helices are proposed to initiate sequential conformational changes in the four domains of the sodium channel, resulting in the transfer of at least four gating charges across the membrane and, finally, in activation of the ion channel. The requirement for sequential conformational changes in the four domains before activation of the ion channel fits with the expected kinetics of sodium channel activation in which gating charge moves immediately on depolarization, whereas ionic current begins to flow only after a significant lag time (13). The presence of four domains in the sodium channel structure that each contains an S4 segment immediately accounts for a gating charge movement of +4. The S4 segments of sodium channels contain enough positively charged residues to allow outward movement of two or more gating charges, resulting in a total possible gating charge movement of +8 by this mechanism.

The sliding helix model is one of the simplest schemes to account for the general properties of voltage-dependent activation and gating currents. It accomodates a unitary gating charge movement of approximately +1 naturally with one ion pair exchange along the sliding helix. However, current estimates of the unitary gating charge movement from fluctuation analysis of the gating current of sodium channels expressed in *Xenopus* oocytes indicates two or three sequential unitary movements of +2.3 gating charges (68). This would require the exchange of two ion pair partners in each unitary gating charge movement in a sliding helix model. A more complex "propagating helix" model designed to accomodate the time course and quantal size of gating current more closely suggests that the S4 segments are part helical and part β-sheet in their resting configuration (103). Depolarization causes a transition from α-helix to β-sheet and a bending of the S4 segment leading to propagation of the helix outward across the membrane. The propagation and bending motions serve to move ~2.3 gating charges across the membrane and initiate a conformational change leading to activation. Further experimental results are necessary to provide a clear test of these models of gating.

Direct experimental support for designation of the S4 segments as the voltage sensors for activation of the voltage-gated ion channels has been provided by site-directed mutagenesis experiments on both sodium channels (255) and potassium channels (199). Neutralization of the one to three positively charged amino acid residues in the S4 segment in domain I of the sodium channel α-subunit causes a progressive reduction in the steepness of the voltage-dependent activation of sodium

channels as expected if these positively charged amino acid residues serve as gating charges. The effect of neutralization of different charged residues is not equivalent, indicating that they do not all move through a comparable fraction of the membrane electric field. Because the electric field is not expected to be strictly uniform through the membrane, the relative distance moved by the gating charges cannot be directly inferred from the fraction of the field through which they move, so this value cannot be used to define a detailed molecular mechanism.

If the S4 helices must move through the protein structure of the sodium channel as the channel activates, the mass and shape of the amino acid side chains might affect the voltage dependence of gating by making it easier or more difficult for the gating segments to move. In fact, mutation of positively charged amino acid residues in S4 helices from arginine to lysine, which retains positive charge, can cause a large shift in the voltage dependence of channel activation (255). Moreover, the shifts in voltage dependence of activation caused by mutation of arginine residues to uncharged glutamine residues are not precisely correlated with the number of charges neutralized, suggesting that mass and shape of the residues may also be important (255). In addition, mutation of a hydrophobic residue in the S4 segment in domain II from leucine to phenylalanine causes a 20-mV shift in the voltage dependence of gating to more positive membrane potentials (16). These effects are expected if these segments must move through the channel structure, as suggested in the sliding helix or propagating helix models of voltage-dependent gating. Overall, these mutational analyses provide strong evidence that the S4 segments are indeed the voltage sensors of the voltage-gated ion channels. The results are consistent with either a sliding helix or propagating helix mode of gating but do not yet prove either of these mechanisms.

2. *Inactivation*

The sodium channel segments that are responsible for other aspects of channel function have not been as easily identified by inspection of the amino acid sequence of the α-subunit. In one approach to identifying domains that are important for inactivation, a panel of site-directed antipeptide antibodies was prepared against peptides corresponding to short (~20 residues) segments of the α-subunit and applied to the intracellular surface of the sodium channel from the recording pipette in whole cell voltage-clamp experiments or from the bathing solution in single-channel recording experiments in excised, inside-out membrane patches (268, 269). In both cases, only one antibody, directed against the short intracellular segment connecting homologous domains III and IV (*h*; see Fig. 4), inhibited sodium channel inactivation. Inhibition of fast sodium channel inactivation of antibody-modified sodium channels in membrane patches was complete. The binding and effect of the antibody were voltage dependent. At negative membrane potentials where sodium channels are not inactivated, the antibody bound rapidly and inhibited channel inactivation; at more positive membrane potentials where the sodium channel is inactivated, antibody binding and action were greatly slowed or prevented. On the basis of these results, it was proposed that the segment that this antibody recognizes (see Fig. 4) is directly involved in the conformational change leading to channel inactivation. During this conformation change, this inactivation gating segment is proposed to fold into the channel structure, serve as the inactivation gate by occluding the transmembrane pore, and become inaccessible to antibody binding (268, 269).

A similar model is supported by site-directed mutagenesis experiments (255). Expression of the sodium channel α-subunit in *Xenopus* oocytes as two pieces corresponding to the first three domains and the fourth domain results in channels that activate normally but have slowed inactivation. The physiological characteristics of these cut channels are similar to those of sodium channels with inactivation blocked by the site-directed antibody. In contrast, sodium channel α-subunits cut between domains II and III have normal functional properties. These two independent approaches provide strong support for identification of the short intracellular segment connecting domains III and IV as an inactivation gating loop.

The inactivation gating loop contains highly conserved clusters of positively charged and hydrophobic amino acid residues. Neutralization of the positively charged amino acid residues in the inactivation gating loop of the sodium channel by site-directed mutagenesis does not have a profound effect on channel inactivation (174), although neutralization of the cluster of positively charged residues at the amino-terminal end of the loop does slow inactivation and shift the voltage dependence of both activation and inactivation. In contrast, deletion of the 10-amino acid segment at the amino-terminal end of the loop completely blocks fast sodium channel inactivation. Mutation of the three-residue hydrophobic cluster IFM to glutamine completely blocks fast sodium channel inactivation (Fig. 6; 277). The single phenylalanine in the center of this cluster is the critical residue. Conversion of it to glutamine is sufficient by itself to nearly completely prevent fast channel inactivation. Mutation of the adjacent isoleucine and methionine to glutamine also has profound effects. On the basis of these results, it has been proposed that these residues serve as the inactivation gating particle of Hodgkin and Huxley (125), entering the intracellular mouth of the transmembrane pore of the sodium channel and blocking it during channel inactivation. The intracellular loop between domains III and IV therefore serves as an "inactivation gate" and closes the transmembrane pore of the sodium channel from the intracellular side of the membrane. This inactivation gating loop of the sodium channel is proposed to function similarly to "hinged-lid" structures in allosteric enzymes that control substrate access to active sites (276). The

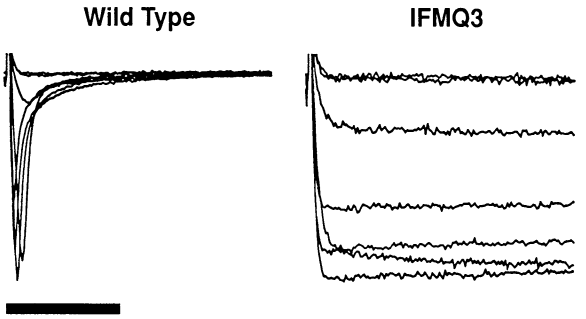


FIG. 6. Inactivation of wild-type and mutant sodium channels. RNA encoding wild-type or IFMQ3 sodium channel α-subunits was transcribed in vitro and injected into *Xenopus* oocytes together with RNA encoding β1-subunits. Sodium currents expressed in oocytes were recorded by whole cell voltage clamp using 2-microelectrode voltage-clamp procedure (196). Sodium currents were elicited by voltage steps from a holding potential of −100 mV to test potentials of −50 to 0 mV in 10-mV increments. Calibration bar is 20 ms.

hydrophobic cluster IFM may function as a molecular "latch" to hold the hinged lid closed.

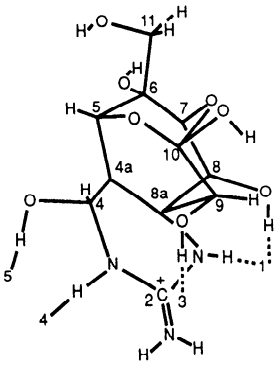
3. *Receptor site for α-scorpion toxins*

Inactivation of sodium channels is slowed by numerous neurotoxins, including the α-scorpion toxins that act at neurotoxin receptor *site 3* on the extracellular aspect of the sodium channel (see Table 1). Binding of the α-scorpion toxins to this receptor site is strongly voltage dependent, suggesting that the voltage-dependent conformational changes that lead to channel activation and inactivation involve channel segments that also contribute to the formation of the α-scorpion toxin receptor site (52, 54). Photoreactive derivatives of α-scorpion toxins specifically label both the α- and β1-subunits of sodium channels (32, 237). The site of covalent labeling has been identified by cleavage of the channel with proteases or cyanogen bromide followed by immunoprecipitation of the resulting labeled peptide fragments with site-directed antibodies (261). The site of covalent attachment is located in the proposed extracellular loop between transmembrane segments S5 and S6 in domain I of the α-subunit (see Fig. 4). To identify other channel segments that may associate to form the α-scorpion toxin receptor site, the effects of several antibodies directed against the proposed extracellular loops of the sodium channel α-subunit were examined for their ability to inhibit binding of α-scorpion toxins to the channel (263). Antibodies that recognize the extracellular loop between segments S5 and S6 in domain I (see Fig. 4) were effective inhibitors of binding as expected. Surprisingly, an antibody directed against the corresponding loop on the extracellular side of segment S5 in domain IV was also an effective inhibitor of toxin binding. Although these two segments are distant in the primary structure of the α-subunit, it is assumed that the four homologous domains of the sodium channel form a square array in the membrane with a transmembrane pore in their center. In this case, domains I and IV are expected to be close to each other in the tertiary structure of the channel and may form the receptor site for α-scorpion toxins by the close apposition of segments of the extracellular loops between transmembrane segments S5 and S6 of both domains I and IV. Binding of α-scorpion toxins to this receptor site slows the coupling of activation to inactivation, implicating channel structures in this region in the coupling of these gating processes.

4. *Receptor site for blockers of sodium channels*

Electrophysiological studies have shown that tetrodotoxin and saxitoxin modify the functional properties of sodium channels when applied to the external surface of the cell (117, 120, 179, 210). These results place neurotoxin receptor *site 1* on the extracellular surface of the channel. Several lines of evidence from a combination of voltage-clamp and chemical modification studies implicated an essential carboxyl group in the tetrodotoxin/saxitoxin receptor site on the sodium channel. Tetrodotoxin and saxitoxin binding are blocked by protonation of a group with a pK_a of ~5.4 in a number of experimental systems (20, 114, 209, 272). Toxin binding is also blocked by treatment of excitable membranes with carboxyl-modifying reagents such as carbodiimides followed by a nucleophile (242) or trialkyloxonium salts (19, 209). These chemical modifications seem specific, since the irreversible block of toxin binding is prevented if the reactions are carried out in the presence of saturating concentrations of tetrodotoxin. Sodium channels made tetrodotoxin insensitive by these chemical reactions are still active in generating action potentials (18, 19, 242) and have normal voltage dependence of activation and inactivation and normal ion selectivity in voltage-clamp studies (248). However, modified sodium channels having this carboxyl group methylated by trimethyloxonium have only 35% of the maximum sodium transport rate of normal channels (246). Thus this carboxyl group is not essential for ion transport, but it is required for binding of tetrodotoxin and saxitoxin, and the charged form is necessary for achieving maximum transport rate.

As described in section IV*B*, tetrodotoxin inhibits purified sodium channels from eel electroplax that contain only an α-subunit, derivatives of tetrodotoxin can be covalently attached to that protein in a specific affinity labeling reaction (159), and sodium channels expressed from α-subunit cDNA are inhibited by tetrodotoxin. These results show that neurotoxin receptor *site 1* is on the α-subunit of the channel. This receptor site is widely considered to be located near the extracellular end of the transmembrane pore of the sodium channel such that binding of the cationic toxins at that site impedes access of transported monovalent cations to the pore (120, 179, 210). Because the toxin has a guanidino moiety and guanidine is a permeant ion of the sodium channel, it has been proposed that the toxin enters the



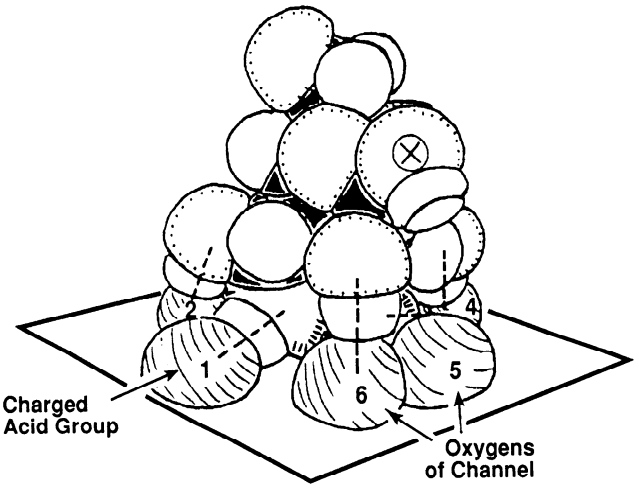


FIG. 7. Model of tetrodotoxin receptor site at ion selectivity filter of sodium channel is illustrated as proposed by Hille (120) on basis of chemical considerations and studies of ion selectivity and toxin block. Space-filling model of toxin (*right*) corresponding to its chemical structure (*left*) is illustrated positioned over a rectangular ring of oxygen atoms in receptor site at mouth of sodium channel. Interactions between each of oxygen atoms surrounding mouth of transmembrane pore and groups on toxin molecule are depicted.

extracellular opening of the transmembrane pore of the sodium channel and lodges there (Fig. 7). If this model is correct, identification of the amino acid residues that are required for binding tetrodotoxin should also give insight into the regions of the channel structure that form the extracellular opening of the pore.

Neutralization of Glu^{387} by site-directed mutagenesis and expression of the modified channels in *Xenopus* oocytes causes a complete loss of tetrodotoxin inhibition of the expressed sodium channels (183). This residue is located just outside transmembrane segment S6 in domain I of the sodium channel (see Fig. 3). The corresponding residues in the other domains are also negatively charged, and neutralization of them by site-directed mutagenesis also dramatically reduces tetrodotoxin binding (262; Fig. 4). Presumably, treatment of sodium channels with trimethyloxonium methylates one or more of these carboxyl groups and causes a reduction of tetrodotoxin binding and ion conductance. In addition, Tyr^{374} in skeletal muscle sodium channels is also required for the high-affinity binding of tetrodotoxin (228). It is located two residues from the required negatively charged residues in the first domain. This residue is changed to Cys in cardiac (h1) sodium channels, causing them to have 200-fold reduced affinity for tetrodotoxin compared with muscle sodium channels (228). Evidently, these residues in analogous positions in each domain form a single binding site for tetrodotoxin that is in or near the extracellular end of the transmembrane pore of the sodium channel. This region may contribute to formation of both the tetrodotoxin receptor site and the extracellular opening of the transmembrane pore.

5. *Transmembrane pore*

Essentially all models for the structure of the voltage-gated ion channels include a transmembrane pore in the center of a square array of homologous transmembrane domains. Each domain would contribute one-fourth of the wall of the pore. Identification of the segments of these proteins that line the transmembrane pore and define the single-channel conductance and ion selectivity of the channels is of great interest and importance. Models have been suggested in which one of the six proposed transmembrane α-helices of the sodium channel contribute to forming the walls of the transmembrane pore (101, 180, 181). In addition, short segments (designated SS1 and SS2) between proposed transmembrane α-helices S5 and S6 have also been suggested to be membrane associated and contribute to pore formation (103, 104). Recent studies on sodium, calcium, and potassium channels all support proposals in which the S5 and S6 α-helical segments and the short segments SS1 and SS2 are intimately involved in pore formation. As described in section v*D3*, the receptor site for tetrodotoxin, an extracellular pore blocker of sodium channels, includes acidic amino acid residues immediately on the extracellular side of transmembrane segment S6 in each domain (183, 262). The receptor site for verapamil, a probable intracellular pore blocker of calcium channels, involves residues immediately on the intracellular side of transmembrane segment IVS6 (253). These results argue that the ends of S6 segments form part of the intracellular and extracellular openings of the transmembrane pore.

Recent results on potassium channels provide direct evidence that regions analogous to the SS1 and SS2 segments form the lining of the transmembrane pore (199). Site-directed mutagenesis and formation of chimeric potassium channels between isoforms with different ion conductance and pharmacological properties have revealed that several amino acid residues located near the extracellular ends of the S5 and S6 segments are required for block of the channel from the extracellular side by the polypeptide charybdotoxin and by tetraethylammonium ion (for review see Ref. 171). Moreover, residues in the center of the segment containing SS1 and SS2 are important for block of the channel from the intracellular side by tetraethylammonium ion. These results argue that the short segments SS1 and SS2 may traverse the membrane in an extended conformation, placing the residues between them on the intracellular side of the channel. These short segments may therefore form the inner walls of the transmembrane

pore, and the residues between them may form an intracellular binding site for tetraethylammonium ion. Consistent with this idea, minor changes in the amino acids in this segment have dramatic effects on ion selectivity (288, 139). The S5 and S6 segments may surround the inner walls of the pore and form the wider intracellular and extracellular openings through which ions enter and exit.

A key role for residues which bind tetrodotoxin at the extracellular mouth of this putative pore region in determination of ion selectivity of sodium channels is indicated by recent mutagenesis results (113). Mutation of Lys^{1422} and Ala^{1714} (Fig. 4) to negatively charged glutamate residues caused a dramatic change in the ion selectivity of the sodium channel from sodium selective to calcium selective. In addition, these changes created a high-affinity site for calcium binding and block of monovalent ion conductance through the sodium channel, as has been previously described for calcium channels (7, 116). Thus, in the mutant sodium channel with two additional negative charges near the extracellular mouth of the putative pore region, monovalent cation conductance is high in the absence of calcium. At calcium concentrations in the 10 μM range, monovalent cation conductance is strongly inhibited by high-affinity calcium binding. As calcium concentrations are increased, calcium conductance is preferred over sodium conductance. These results mirror the ion conductance properties of calcium channels and indicate that a key structural determinant of the ion selectivity difference between calcium and sodium channels is specified by the negatively charged amino acid residues at the mouth of the putative pore-forming region.

VI. PHYSIOLOGICAL MODULATION OF SODIUM CHANNEL FUNCTION

Physiologists are used to thinking of sodium channels as faithful transmitters of action potentials along axons, a process that is not considered to be physiologically regulated. However, sodium channels in neuronal cell bodies and axon initial segments determine the threshold for action potential generation and influence the frequency of neuronal firing (11, 26, 69, 91, 247). Sustained sodium currents in some projection neurons may affect action potential duration and repetitive firing (250, 251). Sodium channels are also present in nerve terminals (1, 143, 258) and can potentially influence the amount of neurotransmitter released from presynaptic nerve endings. These integrative functions of sodium channels are more likely targets for neuromodulation.

A. *Modulation of Brain Sodium Channels by Adenosine 3′,5′-Cyclic Monophosphate-Dependent Protein Phosphorylation*

The possibility of modulation of sodium channel function by adenosine 3′,5′-cyclic monophosphate (cAMP)-dependent phosphorylation was first suggested by biochemical experiments showing that the α-subunit of the sodium channel purified from rat brain was rapidly phosphorylated by cAMP-dependent protein kinase on at least three sites (71). Sodium channel α-subunits in intact synaptosomes are rapidly phosphorylated in response to agents that increase cAMP, and neurotoxin-activated ion flux through sodium channels is reduced concomitantly (72, 73). Stimulation of rat brain neurons in primary cell culture with agents that increase cAMP also causes rapid phosphorylation of sodium channel α-subunits (219). Substantial phosphorylation is observed at the basal level of cAMP in the cultured neurons, and a twofold increase is observed on stimulation.

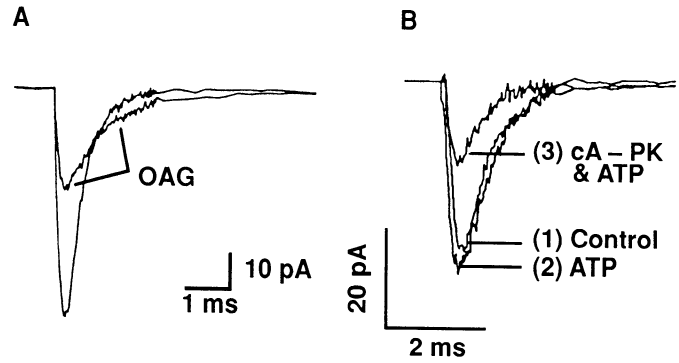


FIG. 8. Differential modulation of sodium currents by protein phosphorylation. *A*: sodium currents were recorded in cell-attached patch configuration in Chinese hamster ovary cells expressing type IIA sodium channel α-subunits during depolarizations from holding potential of −110 mV to test potential of 0 mV. Ensemble average currents were calculated from macropatches containing up to 30 active sodium channels (186). Current traces are illustrated under control conditions and after activation of protein kinase C with synthetic diacylglycerol oleylacetylglycerol (OAG). *B*: sodium currents were recorded from same cells in excised patch-clamp configuration during depolarization from holding potential of −130 mV to test potential of −20 mV. Current traces are illustrated under control conditions, after addition of 1 mM ATP, and after addition of 1 mM ATP and 2 μM cAMP-dependent protein kinase (cA-PK). Ensemble average currents were recorded from macropatches containing ~15 or more active sodium channels (153).

The physiological effect of phosphorylation of sodium channels is revealed most clearly by analysis of the effect of direct phosphorylation of sodium channels in excised membrane patches by purified cAMP-dependent protein kinase (153). Phosphorylation of the inside-out membrane patches from rat brain neurons or transfected CHO cells reduces peak sodium currents ~50% with no change in the time course or the voltage dependence of activation or inactivation of the sodium current (Fig. 8*B*). If the basal activity of cAMP-dependent protein kinase in transfected cells is blocked by coexpression of a dominant-negative mutant form of the regulatory subunit (229), the level of sodium current per expressed sodium channel is increased, indicating that the level of channel activity is subject to tonic modulation at the basal level of activity of cAMP-dependent protein kinase in CHO cells. Phosphorylation of sodium channels in excised membrane patches from these kinase-negative cells by purified cAMP-dependent pro-

tein kinase causes up to 80% reduction of sodium current (Fig. 8). Thus the dynamic range over which sodium channel activity can be modulated by cAMP-dependent phosphorylation is substantial.

The modulation of sodium channel function in intact neuronal preparations, such as brain slices, has not yet been studied. However, sodium currents in acutely dissociated striatonigral neurons are reduced by agonists acting at D_1 dopamine receptors, which activate adenylate cyclase, and are increased by agonists acting at D_2 dopamine receptors, which inhibit adenylate cyclase (255a). Neither the time course nor the voltage dependence of the sodium current is altered. These results show that neuronal sodium channels can be modulated by neurotransmitters acting through cAMP as a second messenger. This dynamic modulation of sodium channel function by the wide range of neurotransmitters and neuromodulators that alter cAMP levels is expected to have profound effects on the excitability of central neurons.

The sites of phosphorylation of the sodium channel by cAMP-dependent protein kinase have been identified by a combination of two-dimensional phosphopeptide mapping, immunoprecipitation of phosphopeptides with site-directed antipeptide antibodies, and microsequence determination (220, 221). Four sites of in vitro phosphorylation are clustered in the large intracellular loop connecting homologous domains I and II (see Fig. 4). These sites are all phosphorylated in intact neurons, but their different rates of phosphorylation in vitro suggest that a subset of the sites may play a predominant role in channel regulation. Mutation of individual serine residues followed by expression and functional analysis will be required to define the role of each site in regulation by cAMP-dependent phosphorylation.

B. *Modulation of Other Sodium Channels by Adenosine 3′,5′-Cyclic Monophosphate-Dependent Phosphorylation*

The intracellular loop of the α-subunit that is phosphorylated by cAMP-dependent protein kinase is conserved among the types I, II, and III sodium channels that are expressed predominantly in the central nervous system but is highly variable in other sodium channels. Thus sodium channels in other tissues may be differently regulated by cAMP-dependent phosphorylation. The μ1-sodium channel expressed in adult skeletal muscle has no consensus sites for cAMP-dependent phosphorylation in its relatively short loop between domains I and II (266). The μ1-α-subunit is phosphorylated by cAMP-dependent protein kinase in vitro, but its regulation by phosphorylation has not been reported (285). The h1 sodium channel α-subunit expressed in adult heart has consensus phosphorylation sites in the corresponding intracellular loop (213) and is phosphorylated by cAMP-dependent protein kinase in vitro (99). Voltage-clamp studies of cardiac myocytes indicate that the h1 sodium channel is modulated by the action of norepinephrine at β-adrenergic receptors via two parallel pathways. Activation of the guanyl nucleotide regulatory protein G_s itself shifts the voltage dependence of inactivation toward more negative membrane potentials (236). In addition, activation of adenylate cyclase by G_s causes an additional negative shift in the voltage dependence of inactivation through cAMP-dependent phosphorylation (193, 236). Thus, in the heart, cAMP-dependent protein phosphorylation has a negative regulatory influence on sodium channel activity, but the mechanism involves an increase in inactivation at normal resting membrane potentials rather than a simple reduction in peak sodium current elicited from all holding potentials as for brain sodium channels.

C. *Modulation of Sodium Channels by Protein Kinase C*

1. *Mechanism of modulation of brain sodium channels*

The α-subunits of purified sodium channels from rat brain are also phosphorylated by protein kinase C (73, 176), suggesting that they may be modulated by the calcium-diacylglycerol signaling pathway. In agreement with this suggestion, sodium currents in neuroblastoma cells are reduced by treatment with fatty acids that can activate protein kinase C (155), and sodium currents in *Xenopus* oocytes injected with rat brain mRNA are reduced by treatment with phorbol esters that activate protein kinase C (77, 243). Activation of protein kinase C in rat brain neurons or in CHO cells transfected with cDNA encoding the type IIA sodium channel α-subunit by treatment with diacylglycerols causes two functional effects, slowing of inactivation and reduction of peak current (186; Fig. 8*A*). Both of these actions are prevented by prior injection of the pseudosubstrate inhibitory domain of protein kinase C into the cells, indicating they reflect phosphorylation by protein kinase C. Moreover, both effects can be observed by phosphorylating sodium channels in excised, inside-out membrane patches directly with purified protein kinase C (186). These results support the conclusion that protein kinase C can modulate sodium channel function by phosphorylation of the α-subunit of the sodium channel protein itself as observed with purified sodium channels (72, 73).

The intracellular loop connecting domains III and IV has been implicated in sodium channel inactivation as described in section v*D2* (196, 255, 268, 269, 276). This segment has a consensus sequence for phosphorylation by protein kinase C centered at Ser^{1506} (see Fig. 4). Mutagenesis of this serine residue to alanine blocks both of the modulatory effects of protein kinase C (275). Evidently, phosphorylation of this site is required for both slowing of sodium channel inactivation and reduction of peak sodium current by protein kinase C.

Treatment of neurons or transfected cells with increasing concentrations of diacylglycerol reveals a biphasic modulation; low concentrations slow sodium

channel inactivation, whereas higher concentrations are required to cause reduction of peak sodium currents (189). These results suggest that a second site of phosphorylation is required for reduction of peak sodium currents. Because cAMP-dependent phosphorylation of sites in the intracellular loop between domains I and II causes reduction of peak sodium currents, mutant sodium channels with alterations in consensus sequences for protein kinase C phosphorylation in that region of the channel were examined for modulation by protein kinase C. Mutation of a serine residue, located in a protein kinase consensus sequence toward the NH_2-terminal end of this intracellular loop (see Fig. 4), prevented the reduction in sodium current by protein kinase C (189). These results implicate phosphorylation of this residue in the reduction in peak sodium current caused by protein kinase C and suggest that this effect of protein kinase C phosphorylation has the same underlying molecular mechanism as reduction of peak sodium current by cAMP-dependent protein phosphorylation.

Sodium channels in the cell bodies of major projection neurons in the central nervous system generate sustained sodium currents of ~1–3% of the peak sodium current (89, 250, 251). Sustained sodium currents are thought to be important determinants of action potential firing patterns of these central neurons. The central neurons that generate these currents have a specific localization of type I sodium channels in their cell bodies (278). These findings led to the hypothesis that type I sodium channels are responsible for generation of sustained sodium currents. Could modulation by protein kinase C be responsible for generation of sustained sodium currents by type I sodium channels? If so, regulation of the extent of modulation by neurotransmitters acting through the calcium-diacylglycerol signaling pathway would be expected to have an important regulatory influence on the size of these sustained sodium currents and thereby regulate the firing patterns of central neurons.

2. *Modulation of sodium channels by protein kinase C in other cells*

The protein kinase C consensus site at Ser^{1506} is conserved in sodium channels expressed in heart and skeletal muscle (135, 213, 266). As for central neurons, activation of protein kinase C in these cells causes slowing of sodium channel inactivation and reduction of peak sodium current (187). Thus protein kinase C modulation of sodium currents is a common feature of most excitable cells.

D. *Modulation of Sodium Channels by G Proteins*

Voltage-gated calcium and potassium channels are modulated by activation of G proteins through a membrane-delimited pathway that does not require intracellular components or protein phosphorylation (46). Although it has not been widely studied, evidence suggests that cardiac sodium channels can also be modulated by activation of β-adrenergic receptors and G_s through a membrane-delimited pathway (236).

VII. BIOSYNTHESIS, POSTTRANSLATIONAL PROCESSING, AND ASSEMBLY OF SODIUM CHANNELS

A. *Glycosylation of Sodium Channel Subunits*

Solubilized sodium channels are specifically adsorbed to and eluted from lectin-Sepharose columns, indicating that they are glycoproteins (23, 108, 132). The α-subunits from electroplax (172), brain (85, 102, 167), and skeletal muscle (212) are glycosylated. Twenty-nine percent of the mass of the electroplax protein is carbohydrate (172), whereas 20–30% of the rat brain and skeletal muscle α-subunits is carbohydrate (102, 167, 212). The β1- and β2-subunits of the sodium channel from mammalian brain specifically bind wheat germ agglutinin and are also heavily glycosylated with 30–36% of their mass as carbohydrate (102, 167, 212). The apparent masses of the deglycosylated subunits are as follows: α, 200 kDa for electroplax, 209 kDa for skeletal muscle, or 220 kDa for rat brain; β1, 23 kDa for rat brain, 26 kDa for skeletal muscle; and β2, 21 kDa for rat brain.

The carbohydrate moieties of the eel electroplax and rat brain sodium channels include long chains of poly-(2,8)-linked sialic acid groups, an unusual posttranslational modification of vertebrate proteins (131, 289). These sialic acid chains contribute ~60 kDa to the apparent mass of the electroplax sodium channel protein.

Removal of the sialic acid chains from purified sodium channels from eel electroplax and rat brain alters their functional properties recorded after reconstitution in planar phospholipid bilayers (206, 232). Discrete subconductance states with conductances of approximately one-half and one-fourth of the normal value are observed with much greater frequency, although the normal conductance state is still the most common one observed. In addition, the voltage dependence of activation of the batrachotoxin-modified electroplax sodium channel is more variable, and the mean voltage dependence is substantially shifted (206). The effects on subconductance states seem likely to result from destabilization of the functional states of the sodium channel by removal of sialic acid. The shift in voltage dependence may result from a similar destabilization or from a change in the surface charge on the extracellular surface of the sodium channel due to the removal of the large negative charge of the sialic residues.

B. *Other Posttranslational Modifications*

The sodium channels from eel electroplax and brain are also subject to modification by fatty acylation and

sulfation of their α-subunits (150, 234). The purified electroplax sodium channel has ~25 mol of covalently attached fatty acid (150). Radiolabeled palmitate is incorporated into the rat brain sodium channels synthesized by brain neurons in cell culture in a thioester linkage to cysteine residues (234). Radiolabeled sulfate residues are also incorporated into sodium channel α-subunits (234). Incorporation of sulfate is inhibited by tunicamycin, an inhibitor of *N*-linked glycosylation, suggesting that the sulfate residues are present on sulfated carbohydrate chains.

C. Biosynthesis of the Sodium Channel

In mammalian brain, the sodium channel is a heterotrimeric complex of three glycoprotein subunits, and the α- and β2-subunits are linked by disulfide bonds. The three subunits must therefore undergo several steps of posttranslational processing before a structurally mature sodium channel can be produced (Fig. 9). Initial evidence for the importance of these posttranslational processing events in channel biosynthesis was derived from studies of the effects of tunicamycin on sodium channel levels in cultured neuroblastoma cells (270) and skeletal muscle cells (27). Inhibition of *N*-linked protein glycosylation with tunicamycin reduces the level of functional sodium channels as measured by saxitoxin binding to <20% of normal in 48 h of treatment. The number of saxitoxin binding sites is reduced, with a half-life of 18–22 h, suggesting that the mature sodium channels present on the cell surface before treatment with tunicamycin have a half-life in this range.

The biosynthesis and the precursor forms of the sodium channel in primary cultures of embryonic rat brain neurons have been studied with isotopic labeling methods and specific antisera directed against the α-subunit (233–235). Labeling of the entire sodium channel population of embryonic rat brain neurons with [γ-^{32}P]ATP and cAMP-dependent protein kinase followed by immunoprecipitation reveals that approximately two-thirds of the α-subunits in these cells, and in neonatal rat brain in vivo, are not linked to β2-subunits by disulfide bonds (235). Further analysis showed that these free α-subunits are full sized, membrane associated, and have complex carbohydrate chains like mature α-subunits. However, they are located in the intracellular compartment and do not bind saxitoxin with high affinity. Free α-subunits are not observed in adult rat brain. Thus it was proposed that they form an inactive reserve of sodium channels in developing neurons for incorporation into the cell surface during periods of rapid membrane assembly in development (233).

The first form of the newly synthesized α-subunit, detected after a 5-min pulse of [^{35}S]methionine, has an apparent mass of 224 kDa (233). In the presence of tunicamycin to block cotranslational glycosylation, the newly synthesized α-polypeptide has an apparent mass of 203 kDa. The amino acid sequence of the α-subunit

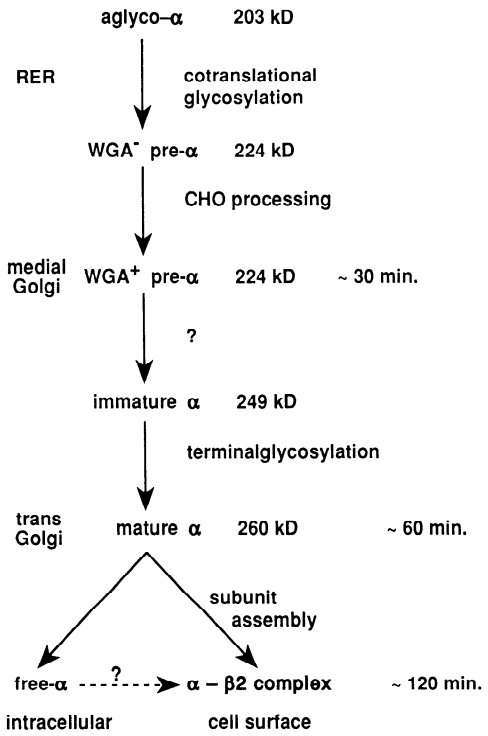


FIG. 9. Steps in biosynthesis of sodium channel in brain neurons. aglyco-α, unglycosylated α-subunit; CHO, carbohydrate; RER, rough endoplasmic reticulum; WGA, wheat germ agglutinin.

does not reveal a cleavable hydrophobic leader sequence. Consistent with this, the mass of this initial translation product increases progressively during further processing. Over 2 h, this initial precursor undergoes a single-step increase in apparent mass to 249 kDa followed by a slow increase to the mature apparent mass of 260 kDa. During this time, the carbohydrate chains are processed to give complex structures containing *N*-acetylglucosamine and sialic acid, as indicated by binding to wheat germ agglutinin. After 1 h, newly synthesized α-subunits begin to be linked to β2-subunits by disulfide bonds, and this process continues for ~8 h until an average maximum of one-third of the newly synthesized α-subunits have been linked to β2-subunits (233, 234). Only α-subunits disulfide linked to β2-subunits appear on the cell surface, suggesting that disulfide bond formation is a late event in sodium channel processing and assembly. Because two-thirds of newly synthesized α-subunits do not form a disulfide bond with β2 and remain in an inactive intracellular pool, it appears that posttranslational processing and assembly are rate-limiting steps in the biosynthesis of functional cell surface sodium channels in developing brain neurons.

Multiple steps of posttranslational processing have also been resolved for synthesis of electroplax sodium channels in electrocytes or for expression from electroplax mRNA injected into *Xenopus* oocytes (264). In electrocytes, the initial translation product has an apparent mass of 200 kDa and is processed through several intermediate mass forms to a mature mass of 290 kDa. The intermediate forms are thought to represent different

stages of posttranslational processing and addition of fatty acid and carbohydrate moieties. A similar sequence of mass forms is observed for synthesis of the electroplax sodium channel in the *Xenopus* oocyte, but only a small fraction of the newly synthesized sodium channels were processed to the mature mass in the oocyte. Evidently, there are posttranslational processing reactions for electroplax sodium channels that are specific to the eel electrocyte.

D. Role of β1- and β2-Subunits in Posttranslational Assembly of Sodium Channels

The role of β1- and β2-subunits in sodium channel assembly can be probed critically in studies of the biosynthesis of sodium channels directed by cloned cDNAs in heterologous cells. In *Xenopus* oocytes, α-subunits can be successfully expressed alone, but coexpression of α- and β1-subunits modifies the inactivation properties of the expressed sodium channels and increases their peak sodium current (129). These results suggest that β1-subunits not only modify the functional properties of sodium channels but also increase the efficiency of their expression. However, peak sodium currents could also be increased by modification of the time course of sodium channel gating so that more of the channels open simultaneously during depolarizing pulses. A more direct test of the effect of β1-subunits on expression of sodium channels is provided by mammalian cell expression systems in which the expressed protein can be directly detected by neurotoxin binding and immunoblotting methods. Coexpression of β1-subunits with α-subunits in CHO cells causes a severalfold increase in sodium channel expression with essentially no change in functional properties (L. L. Isom, T. Scheuer, and W. A. Catterall, unpublished observations). Whole cell voltage-clamp current and saxitoxin binding sites are increased comparably. The cell surface density of sodium channels in these transfected cells exceeds the density in brain neurons. The results indicate that assembly with β1-subunits has a dramatic effect on sodium channel assembly in cells in culture and raise the possibility that assembly with β1-subunits can be a rate-limiting process for sodium channel biosynthesis and assembly in vivo.

VIII. LOCALIZATION OF SODIUM CHANNELS IN EXCITABLE CELLS

The functional properties of excitable cells are determined to a substantial extent by the density and localization of sodium channels in their cell surface membranes. In neurons, the distinct functional properties of cell bodies, axons, and dendrites depend critically on differential distribution of sodium channels. The mode of impulse conduction in axons, continuous versus saltatory, depends on the distribution of sodium channels along the axon. Axon initial segments and terminals also have specialized properties that depend on sodium channels. Differences in sodium channel density have been inferred from measurements of local sodium current densities and determined directly by autoradiographic or spectrofluorometric localization of bound toxins or by immunocytochemical localization of bound antibodies. The results of these different localization methods agree well in general and show that sodium channels are strikingly concentrated in some regions of excitable cells.

A. Neurons

1. Axon initial segments

In most central neurons, action potentials are thought to be initiated at the initial segment of the axon. Classic electrophysiological studies show that the threshold for generation of action potentials is lowest at this site (11, 26, 69, 91, 247). The low threshold for action potential generation at the initial segment could in principle results from differences in local passive membrane properties, local potassium permeability, or local sodium channel density. Direct evidence for a high density of sodium channels in axon initial segments comes from experiments localizing bound neurotoxins on neurons in cell culture and from immunocytochemical studies of sodium channel distribution in vivo. Autoradiographic studies of specifically bound ^{125}I-labeled α-scorpion toxin show that the initial segment of neurites of spinal cord neurons in culture has a severalfold higher density of sodium channels than the adjacent cell body (40, 56; Fig. 10). Labeling sodium channels in cultured neurons with fluorescent scorpion toxin derviatives gives similar results (9).

Localization of sodium channels in neurons in vivo by neurotoxin binding or immunocytochemistry is difficult because of the complicated architecture of most neural tissues. Axons having high densities of sodium channels grow over and form synapses on the cell bodies and dendrites of most neurons, obscuring the density of sodium channels on the postsynaptic neuronal membrane. The retina provides an advantageous neural tissue because the cell bodies and synapses of its neurons are segregated into layers. Determination of sodium channel distribution in the retina with an antibody against rat brain sodium channels revealed that only the ganglion cell axon layer had a high density of immunoreactive sodium channels (281). Closer analysis showed that there was a high density of sodium channels in the axon initial segments compared with the adjacent cell body. The density of sodium channels increased abruptly at the axon hillock, forming a clear line of demarcation between it and the adjacent cell soma. Together with the results on neurons in cell culture, the in vivo studies demonstrate that a high density of sodium channels is present at the axon initial segment. These results support the conclusion that increased so-

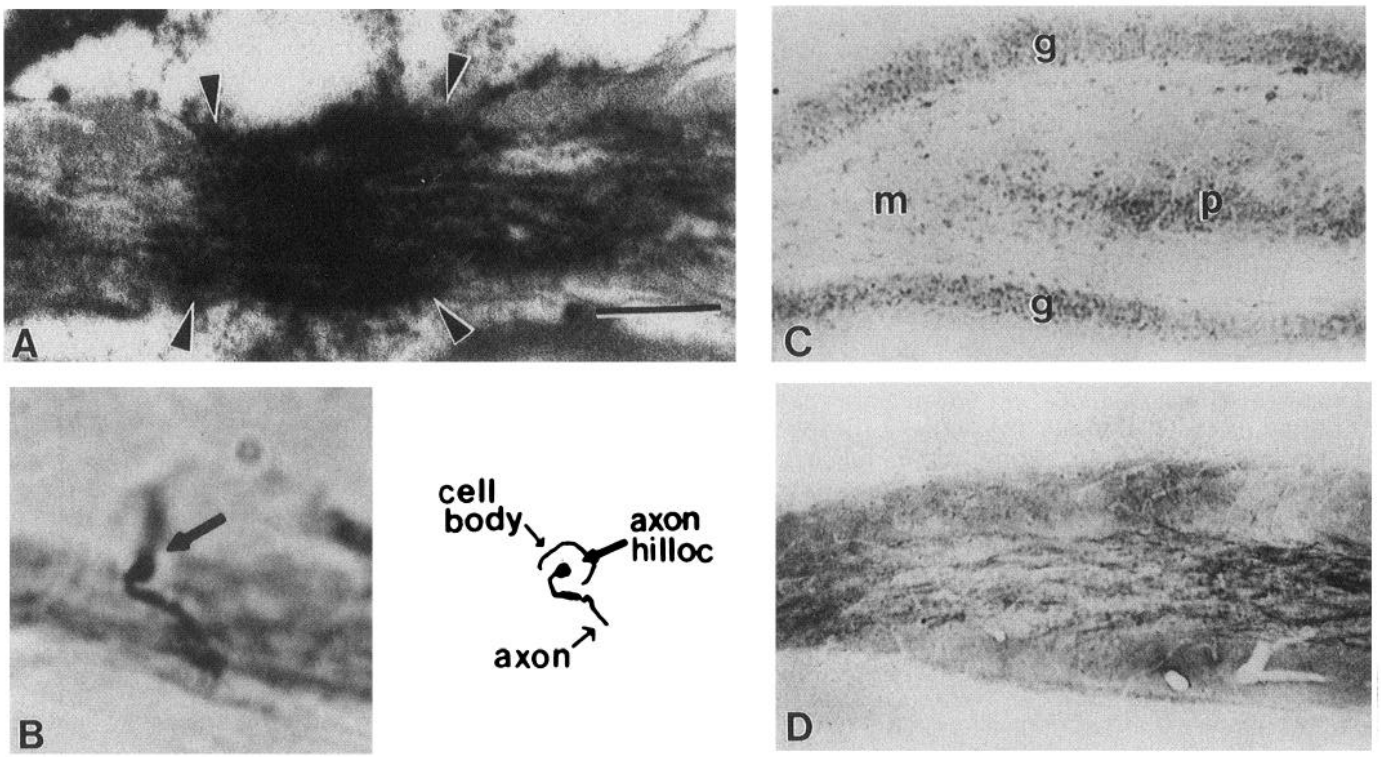


FIG. 10. High-density localizations of sodium channels. *A*: node of Ranvier in rat optic nerve stained with a polyclonal antibody against sodium channel α-subunits and visualized with the biotin-avidin-horseradish peroxidase method. [Adapted from Black et al. (37), which gives experimental details.] Calibration bar, 0.25 μm. *B*: axon hillock and axon initial segment of rat retinal ganglion cell stained with a polyclonal antibody against sodium channel α-subunits and visualized with peroxidase-antiperoxidase method. [Adapted from Wollner and Catterall (281), which gives experimental details.] *C*: sagittal section through hippocampus and dentate gyrus of rat brain stained with a site-directed anti-peptide antibody, which specifically recognizes type 1 sodium channel α-subunits. Cell bodies of dentate granule neurons (g) and CA3 hippocampal pyramidal neurons (p) are stained, but mossy fiber tract (m) containing axons of dentate granule neurons, which form synapses on the CA3 hippocampal pyramidal cells is not stained. [Adapted from Westenbroek et al. (278), which gives experimental details.] *D*: adjacent sagittal section through hippocampus and dentate gyrus of rat brain stained with a site-directed anti-peptide antibody, which specifically recognizes type II sodium channel α-subunits. Mossy fiber tract containing axons of dentate granule neurons, which make synapses on the CA3 pyramidal cells is stained, but cell bodies of dentate granule neurons and hippocampal pyramidal cells are not.

dium channel density is the main reason for the low threshold for action potential generation at the initial segment.

2. *Nodes of Ranvier*

Large sodium currents are generated at nodes of Ranvier to produce sufficient current to activate the sodium channels at the next node and allow saltatory conduction. The measured sodium currents are consistent with approximately 2,000 sodium channels/μm^2 in nodal membrane (67, 271). The internodal axonal membrane under the myelin sheath is electrically insulated from the extracellular fluid, preventing generation of ionic currents. In addition, the internodal axonal membrane has few if any sodium channels. Comparison of high-affinity saxitoxin binding to intact or disrupted nerves containing myelinated axons indicates that disruption of the myelin sheath does not lead to the appearance of new saxitoxin binding sites, indicating that they are all available in the nodes in the intact nerve (210, 271). Immunocytochemical analysis of sodium channel distribution in intact and focally demyelinated nerve fibers shows that immunoreactive sodium channels are confined to the nodal membrane (37, 38, 84). These results indicate that the specialized properties of nodes of Ranvier in saltatory conduction of nerve impulses are due in large part to specific localization of sodium channels within them.

3. *Nerve terminals*

Nerve terminals are specialized for transduction of the depolarization of the action potential into the rapid release of neurotransmitter. Sodium channels are not essential for this process. Nevertheless, several observations indicate that there is a significant density of sodium channels in nerve terminals. Purified brain synaptosomes have sodium channels as determined by ion flux measurements or neurotoxin binding (1, 39, 41, 143, 204, 258). Extracellular recordings detect substantial inward sodium currents as action potentials invade the elongated presynaptic terminals at the frog neuromuscular junction (161). Thus it appears that sodium chan-

nels are not excluded from nerve terminals. In contrast, it seems likely that the action potential is conducted actively into presynaptic terminals by sodium channels and that their activity can influence the extent of depolarization of the nerve terminal and therefore transmitter release. Modulation of sodium channel function in nerve terminals by protein phosphorylation or G proteins may contribute to regulation of synaptic function.

B. Skeletal Muscle

As in the axon initial segments of neurons, sodium channels are concentrated at the site of action potential initiation in skeletal muscle. During synaptic transmission, muscle excitation is initiated by binding of acetylcholine to nicotinic acetylcholine receptors at the postsynaptic muscle endplate and activation of the ion channel of the receptor. The resulting local depolarization activates sodium channels and begins the muscle action potential. Measurements of local sodium currents along the muscle fiber using a loose patch-clamp method revealed a severalfold higher density of sodium current in the periendplate region (29, 48, 212a). A high density of sodium channels is also observed by cytochemical staining of sodium channels with antibodies or fluorescently labeled toxins (12, 80, 106). Higher resolution analysis by immunocytochemical staining or autoradiography of specifically bound ^{125}I-labeled α-scorpion toxin and visualization in the electron microscope showed that sodium channels are clustered in the depths of the junctional folds of the postsynaptic membrane below and adjacent to the clusters of acetylcholine receptors at the tips of the folds (42, 87). This local high density of sodium channels undoubtedly contributes to the high safety margin for neuromuscular transmission by causing a low threshold for initiation of the muscle action potential in the subsynaptic membrane.

C. Determinants of Sodium Channel Localization

1. Differential distribution of sodium channel subtypes

Because multiple sodium channel genes are expressed in most excitable cells, a potential mechanism for differential regulation of the density of sodium channels in adjacent areas of the cell surface membrane is differential membrane targeting and distribution of different sodium channel subtypes coupled with differential regulation of transcription of the corresponding genes. This mechanism appears to contribute to the different cell surface density of sodium channels in cell bodies and axons of central neurons. Site-directed antipeptide antibodies directed against variable amino acid sequences in the intracellular loop between homologous domains I and II of the sodium channel recognize the type I and type II sodium channels specifically (98). Immunoprecipitation studies show that the type I and type II sodium channel subtypes account for >90% of the sodium channels in cortical areas and in the cerebellum. Other types of sodium channels are expressed in the midbrain, medulla, and spinal cord. The ratio of type I to type II sodium channels is <0.2 in the cerebral cortex and increases in more caudal areas of the nervous system, exceeding 2.0 in the spinal cord. Immunocytochemical localization of these two channel subtypes in different brain regions reveals differential distribution within individual neurons (278). Type I sodium channels are localized in low density in the cell bodies of major projection neurons, such as the cerebral cortical and hippocampal pyramidal cells, the cerebellar Purkinje cells, and the spinal motor neurons. In contrast, type II sodium channels are localized in higher density in axons and fiber tracts throughout the brain and spinal cord. The segregation of these two channel subtypes to different subcellular compartments allows differential regulation of the sodium channel density in axons and cell bodies by differential regulation of gene expression.

2. Glycosylation

Axons of the neurons from the giant fiber lobe of the stellate ganglion of the squid fuse to give rise to the giant axon that has been studied extensively by voltage-clamp methods. Normally, the cell bodies of these neurons do not contain measurable levels of sodium channels. Isolation of these neurons in vitro without axons leads to synthesis and insertion of functional sodium channels in the cell body (43). If the cells are maintained in vitro long enough to regrow an axon, sodium channels are preferentially targeted to the newly formed axonal membrane (94). Preferential localization of sodium channels in axons is prevented by inhibition of glycoprotein biosynthesis with tunicamycin, an inhibitor of *N*-linked glycosylation, but insertion of sodium channels in cell bodies is not seriously affected. These results indicate that *N*-linked glycosylation is required for normal targeting of newly synthesized sodium channels to their appropriate cellular target in the growing axon in squid neurons. In mammalian neurons, block of *N*-linked glycosylation prevents sodium channel expression on the cell surface entirely (233, 270).

3. Immobilization of sodium channels

Even though differential targeting to specific membrane compartments may be essential for establishment of high-density localizations of sodium channels, it is likely that immobilization by binding to localized components of the cytoskeleton and/or extracellular matrix is necessary to maintain a specific pattern of localization. Several lines of evidence suggest that sodium channels are immobilized. In skeletal muscle fibers, inactivation of sodium channels within a loose patch-recording pipette by flashes of intense ultraviolet light is followed by very slow diffusion of sodium chan-

nels back into the recording area within the patch pipette, suggesting that sodium channels are immobilized in the muscle surface membrane and cannot diffuse into the area of the photobleached patch (254). Sodium channels labeled by fluorescent α-scorpion toxin in cultured muscle cells have a patchy distribution in the surface membrane (8). Recovery from photobleaching of the fluorescent toxin in the patches of high sodium channel density is slow and incomplete, indicating restricted diffusion of sodium channels in the cell surface membrane. Similarly, recovery from photobleaching of fluorescently labeled sodium channels in regions of high density in the initial segments of neurites of neurons in cell culture is also slow and incomplete (9). Together, these results indicate that sodium channels in regions of high cell surface density are partially or completely immobilized. This mechanism likely serves to maintain differential distributions of sodium channels once they have been established.

What cytoskeletal proteins might serve to interact with sodium channels and immobilize them? One important candidate is ankyrin, a component of the spectrin-based submembrane skeleton that is known to make connections between the chloride-bicarbonate exchanger and the cytoskeleton in red blood cells (33). Ankyrin binds to purified sodium channels in detergent solution and in reconstituted phospholipid vesicles (249) and is colocalized with sodium channels at the base of the junctional folds in the postsynaptic muscle membrane (87). Thus, ankyrin fulfills two of the main criteria expected of a protein anchor, binding affinity for sodium channels and colocalization in regions of high density. Different ankyrin subtypes are expressed in the nervous system and are differentially distributed in cell bodies, axon initial segments, and nodes of Ranvier (140), consistent with a role in sodium channel immobilization in neurons as well as skeletal muscle.

In addition to ankyrin, a small (33 kDa) protein in the Triton-insoluble cytoskeletal fraction that has high affinity for sodium channels in gel blot overlay experiments has been described (83). This protein has not yet been purified and characterized from neural tissue.

4. Interaction with glia

Interactions with other cell types may also influence sodium channel expression and distribution. The intimate interaction between the oligodendrocyte and the axon during myelination provides ample opportunity for cell-to-cell signaling, which may regulate sodium channel distribution. Evidence in favor of such a regulatory role for oligodendrocytes comes from studies of the mutant mouse *shiverer* whose axons are hypomyelinated because of a defect in the gene encoding myelin basic protein (211). In the hypomyelinated axons of these mice, there is a sharp increase in sodium channel density that is continuous along the length of the axons (184). This increase is due entirely to an increase in the type II sodium channels in the hypomyelinated axons as assessed by immunocytochemical analysis with subtype-specific antibodies (279). Type I and type III sodium channels are unaffected. Evidently, interactions with the oligodendrocyte that are dependent on myelin basic protein are required for normal regulation of the number of type II sodium channels inserted into the axonal membrane. Loss of these interactions causes a specific increase in the number of type II sodium channels, the channel subtype that is normally targeted to axons.

Consistent with these results on *shiverer* mice, recent studies of the effects of coculture of Schwann cells and dorsal root ganglion neurons indicate that interactions with Schwann cells can induce clusters of sodium chanels along neurites as detected by immunocytochemistry (133). These clusters have not been shown to be located at sites of future formation of nodes of Ranvier, but the induction of sodium channel clusters by Schwann cell interaction suggests that the myelinating glial cells may induce localizations of sodium channels at nodes of Ranvier, as well as inhibiting type II sodium channel expression and localization at internodal regions as observed in the studies of *shiverer* mice.

IX. REGULATION OF SODIUM CHANNEL EXPRESSION

A. Skeletal Muscle

1. Developmental regulation

Muscle cells express both tetrodotoxin-sensitive and tetrodotoxin-resistant sodium channels. The tetrodotoxin-sensitive sodium channels present in adult muscle have a K_D for tetrodotoxin of ~10 nM (22, 210) and are encoded by the μ1-sodium channel gene (266). Tetrodotoxin-resistant sodium channels were first described in denervated skeletal muscle fibers (107, 208). They have a more negative voltage dependence for activation and a smaller single-channel conductance than tetrodotoxin-sensitive sodium channels (274). They have a K_D for tetrodotoxin of ~1 μM (51, 145a, 195) and are not detected in normal adult muscle fibers. They are also essentially insensitive to μ-conotoxin, a peptide channel blocker of tetrodotoxin-sensitive sodium channels (97, 173), and they have relatively high affinity for sea anemone toxins and relatively low affinity for α-scorpion toxins at neurotoxin receptor site 3 (60). Their pharmacological properties are essentially identical to those of cardiac sodium channels (60), and it is likely that they are encoded by the h1 gene that directs the synthesis of sodium channels with similar tetrodotoxin affinity in *Xenopus* oocytes (75, 280).

These two distinct sodium channel subtypes are expressed in sequence in developing mammalian skeletal muscle fibers. Tetrodotoxin- and μ-conotoxin-resistant sodium channels are expressed early in development, reach a peak shortly after birth, and decline to undetectable levels by *day 15* after birth (96). High-affinity saxi-

toxin binding sites and tetrodotoxin- and μ-conotoxin-sensitive sodium currents are detectable at birth and increase 8- to 10-fold to adult levels by 28 days after birth (96, 239).

2. Effects of electrical activity

The number of cell surface tetrodotoxin-sensitive sodium channels, measured as high-affinity saxitoxin binding sites, in cultured rat muscle fibers is regulated by several different kinds of stimuli. The spontaneous electrical activity of the muscle cells causes a feedback downregulation of sodium channel number. Inhibition of spontaneous activity with tetrodotoxin or local anesthetics increases sodium channel number approximately two- to threefold (44, 240). The effect of spontaneous electrical activity is mimicked by elevation of cytosolic calcium by treatment with A23187 or ryanodine, suggesting that it is mediated by the increase in cytosolic calcium accompanying each action potential (44, 240, 241). Elevation of intracellular cAMP also increases the number of sodium channels (241). Both of these changes are observed without changes in channel degradation rate, implying that one or more of the steps in channel biosynthesis and processing must be the point of regulation (241).

Changes in the levels of mRNA encoding sodium channel α-subunits are correlated with the regulation of sodium channel number by electrical activity and cAMP (190). Inhibition of electrical activity increases the α-subunit mRNA level two- to threefold, and an increase in intracellular calcium by treatment with A23187 decreases the α-subunit mRNA level fivefold. Thus there is an overall 10-fold regulation of sodium channel α-subunit mRNA level in response to changes in electrical activity and cytosolic calcium. In addition, increased cAMP increases α-subunit mRNA threefold. These results indicate that the regulation of the number of tetrodotoxin-sensitive sodium channels is due to changes in the level of mRNA encoding the α-subunit. These changes might result from regulation of transcription, mRNA stability, or both.

3. Effects of innervation/denervation

The expression of both tetrodotoxin-sensitive and tetrodotoxin-insensitive sodium channels is modulated by innervation. Early in postnatal development (up to 11 days postpartum), denervation of the developing nerve fibers accelerates the rate of increase of tetrodotoxin-sensitive sodium channels two- to threefold (96, 239), a similar increase in sodium channel number to that observed in cultured muscle cells whose electrical activity is blocked. Because denervation also reduces the electrical activity of the developing muscle fibers by depriving them of neural stimulation, it is likely that this effect is due to the regulation of appearance of the sodium channel mRNA by reduced electrical activity and cytosolic calcium, as has been observed in muscle cells in culture (190). The target of this regulation is likely to be the mRNA encoding the α-subunit of μ1-sodium channels, which increases rapidly at this time of development (286). After 11 days postpartum, denervation reduces the number of tetrodotoxin-sensitive sodium channels up to 50% (96, 239). This change is also accompanied by a corresponding reduction in the level of mRNA encoding the μ1-sodium channel α-subunit (286). Evidently, regulatory influences other than electrical activity and cytosolic calcium dominate the control of expression of tetrodotoxin-sensitive sodium channels in adult muscle. Chemical factors released from the nerve and/or muscle fibers following development of mature synapses are likely candidates for this regulatory role.

Tetrodotoxin-insensitive sodium channels in skeletal muscle are differentially regulated by these same environmental stimuli. Denervation early in development or in adulthood causes an increase in tetrodotoxin-insensitive sodium channels (96, 107, 195, 208). Denervation of adult skeletal muscle causes a marked increase in the level of mRNA encoding the h1 sodium channel α-subunit (286). The cellular signals that cause these denervation-dependent changes in tetrodotoxin-resistant sodium channels have not yet been defined.

B. Neurons

1. Developmental regulation in brain and retina

As in rat skeletal muscle, sodium channels in rat brain and retina are present at 10–20% of their adult level at birth and increase to adult levels over ~28 days postpartum (28, 105, 157, 158, 230, 283). In the retina, most sodium channels are present in the retinal ganglion cells (281), so the development of sodium channels in a single neuronal population can be studied with both immunocytochemical and biochemical techniques. Sodium channel α-subunits can be detected by embryonic *day 14*, only 1–2 days after formation of the first ganglion cells (283). They remain at a low level until birth and then increase dramatically during the first 3 wk of life as synapses are formed between the axons of the ganglion cells and their central targets in the lateral geniculate and inferior colliculus. The large increase in sodium channel α-subunits in the postnatal period is accompanied by a similar increase in functional sodium channels, as measured by high-affinity binding of saxitoxin or electrical recording and by the appearance of β1- and β2-subunits and mature heterotrimeric sodium channel complexes.

In rat forebrain, the increase in sodium channel levels from ~10–20% of maximum to the adult level between birth and 28 days postpartum also is temporally correlated with the period of synaptogenesis and subsequent myelination of newly developed axons (230). The increase in the number of sodium channel α-subunits results primarily from an increase in the level of α-sub-

unit mRNA. Measurement of the rates of α-subunit gene transcription in nuclear run-off assays shows that this increase is due primarily to increased gene transcription. It is accompanied by the appearance of β1- and β2-subunits, as measured by specific antisubunit antibodies (165, 230), and by formation of mature heterotrimeric sodium channel complexes. Thus the time course of events in sodium channel development in the brain and retina seem similar, and the two systems have provided complementary information on this process. In both neuronal populations, subunits appear early in the postmitotic life of the neurons. Several days after their first appearance, sodium channel levels increase dramatically in correlation with synaptogenesis and myelination. The large increase in sodium channel number is driven by increased transcription of sodium channel α-subunit genes and is accompanied by a large increase in the number of β1- and β2-subunits that form heterotrimeric complexes with α-subunits.

The three sodium channel subtypes expressed primarily in neurons (types I, II, and III) are differentially regulated during development in the central nervous system (30, 31, 98; Fig. 11). Type III sodium channels appear first, reaching peak mRNA level late in embryonic life or shortly after birth and declining to low levels by adulthood (31). Type II sodium channel α-subunit mRNA and protein appear at the end of embryonic development and increase markedly from birth to 28 days postpartum (31, 98). The increase in expression of the type II gene is primarily responsible for the dramatic increase in sodium channel levels during postnatal brain development. Type I sodium channel mRNA and protein appear last and increase steadily throughout development into adulthood (31, 98). Type I channels account for <15% of sodium channels in the adult brain but >50% of the sodium channels in the adult spinal cord.

Distinct size forms of the β1-subunit are expressed differentially during forebrain development (165). A small, 26-kDa form is prominent in embryonic brain but is replaced by the adult 36-kDa form progressively following birth. The embryonic form of the β1-subunit may interact specifically with the type III α-subunits that are expressed primarily during embryonic life.

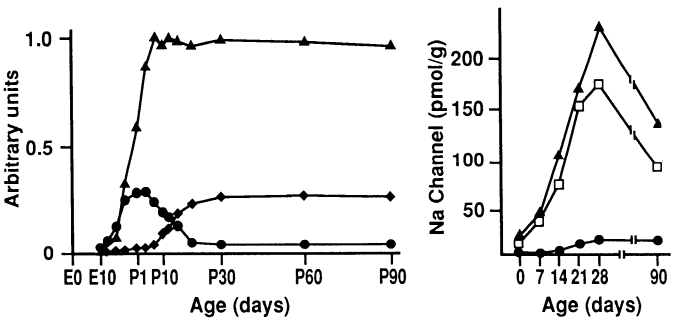


FIG. 11. Time course of development of sodium channel subtypes in brain. *Left*: levels of mRNA measured by Northern blotting methods using cDNA probes specific for type I (diamonds), type II (triangles), and type III (circles) sodium channel subtypes (31). *Right*: levels of type I (filled circles), type II (open squares), and total (filled triangles) sodium channels as measured by immunoprecipitation with subtype-specific antibodies and radiolabeling by protein phosphorylation (98).

2. *Regulation by nerve growth factor*

Nerve growth factor (NGF) has profound effects on developing neurons in the sympathetic nervous system as well as neurons in sensory ganglia and brain. It is required for neurite outgrowth in vitro and for survival of cultured sympathetic neurons. The pheochromocytoma cell line PC12 differentiates into sympathetic neuronlike cells during growth in NGF (100). Differentiation and neurite outgrowth is accompanied by an increase in sodium channel number and in sodium current (78, 162, 192, 224). Under some conditions, the increase in sodium channels caused by NGF can be mimicked by elevation of cellular cAMP and blocked by inhibition of cAMP-dependent protein kinase (136). In other experiments, cAMP appeared not to be essential for the effect of NGF (198). Ribonuclease protection studies indicate that undifferentiated PC12 cells express the type II sodium channel gene at low levels and that the expression is substantially increased after treatment with NGF (162). Evidently, NGF is a potentially important factor in regulating the increase in sodium channel gene expression that occurs during neuronal differentiation. Related growth and differentiation factors specific for other classes of neurons may serve a similar role. Because NGF and related neurotrophic factors are made in target cells of neurons and taken up by nerve terminals at synapses, an important role for them in activating sodium channel gene expression would explain the temporal correlation of the increase in sodium channel density in retina and forebrain with synaptogenesis in those regions of the central nervous system (230, 283).

3. *Tissue-specific gene expression*

In addition to regulation during development, the specific expression of distinct sodium channel α-subunit genes in different tissues implies tissue-specific regulation of gene expression. The mechanisms that regulate tissue-specific transcription and expression of the sodium channel genes have not yet been investigated extensively. However, the available results indicate that the type II gene is expressed primarily in the nervous system because of a strong negative regulatory element in its 5′ untranslated region (163). In cell lines of neural and nonneural origin, a region of 5′ flanking DNA from the transcription start site to nucleotide 1051 was sufficient to silence expression of a reporter gene in transfected muscle cells and other nonneuronal cell lines. Regulation of gene transcription by negative enhancer elements is an unusual mechanism for tissue-specific gene regulation. It will be of interest to determine whether the other brain sodium channel genes are similarly regulated.

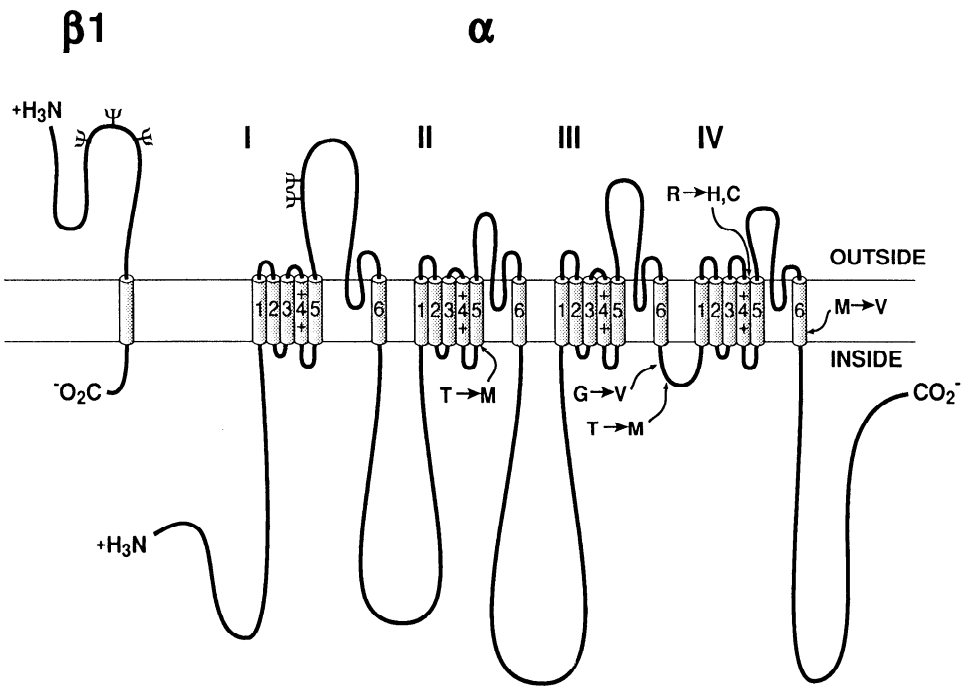


FIG. 12. Sites of mutations in periodic paralyses. Transmembrane folding model of skeletal muscle sodium channel is illustrated with mutations that give rise to hyperkalemic periodic paralysis and paramyotonia congenita and their approximate places in sequence indicated. Individual mutations are described in text.

X. NATURALLY OCCURRING MUTATIONS IN HUMAN SODIUM CHANNEL GENES

A. *Hyperkalemic Periodic Paralysis*

An exciting new development in the biology of sodium channels is the discovery of mutations that cause human muscle disease. Hyperkalemic periodic paralysis is an autosomal dominant disorder characterized by episodic muscle weakness associated with mild elevation of serum potassium (222). Electrophysiological recordings of acutely dissociated muscle from patients with hyperkalemic periodic paralysis show persistent depolarization of the fibers and an abnormal, noninactivating sodium conductance that is activated above −60 mV and blocked by tetrodotoxin (148, 149). Whole cell voltage clamp in normal physiological solution does not reveal any major abnormality (222), but single-channel analysis shows that elevated external potassium causes episodes of repetitive single-channel openings with low probability (49). This behavior is sufficient to cause a sustained sodium current during episodes of late channel openings.

The genetic locus responsible for hyperkalemic periodic paralysis is tightly linked to the muscle sodium channel μ1-α-subunit gene on human chromosome 17 (88, 201). Analysis of the nucleotide sequence of sodium channel genes reveals that multiple defective sodium channel alleles are responsible for the disease in different kindreds (200, 214). These missense mutations include substitution of valine for methionine near the intracellular end of transmembrane segment IVS6 and substitution of methionine for threonine and the intracellular end of transmembrane segment IIS5 (Fig. 12). Evidently, these mutations can cause subtle changes in the inactivation gating properties of sodium channels, even though they are located at a substantial distance from the inactivation gating segment in the primary structure of the sodium channel α-subunit.

B. *Paramyotonia Congenita*

Paramyotonia congenita is a related disorder of periodic muscle paralysis induced by cold exposure and by exercise in the cold (222). Changes in serum potassium are not generally correlated with periods of muscle weakness. Paramyotonia congenita maps to the same muscle sodium channel gene locus on chromosome 17 as hyperkalemic periodic paralysis (201). Analysis of the DNA sequence of mutations in different kindreds also reveals multiple alleles. Two mutations cause substitutions of histidine or cysteine for arginine at the extracellular end of transmembrane segment IVS4, a voltage-sensing segment of the sodium channel (200). Two distinct mutations cause substitutions of methionine for threonine and valine for glycine in the intracellular loop between domains III and IV, the inactivation gating segment of the sodium channel (164).

C. *Molecular Mechanisms of Periodic Paralyses*

The genetic results establish the skeletal muscle sodium channel as the locus of the genetic defects causing most of the inherited periodic paralyses of human skeletal muscle. Although the molecular events through which these mutations cause sodium channel dysfunction remain unknown, comparison of the location of the mutations with the known functional regions of the sodium channel allows some suggestions for molecular mechanisms. All the periodic paralyses seem likely to

alter the process of sodium channel inactivation, as has been demonstrated directly for hyperkalemic periodic paralysis (49). Two paramyotonia congenita mutations are located right within the inactivation gating loop near residues that are essential for inactivation and are likely to form the inactivation gate itself. Therefore these mutations likely intefere with inactivation gating directly by altering the structure of the inactivation gate itself and impairing its closure. The two paramyotonia congenita mutations at the extracellular end of segment IVS6 neutralize a potential gating charge in the voltage-sensing S4 segment. If the S4 segment in domain IV plays a key role in coupling of activation to inactivation, a mutation at this site would be expected to modify the voltage dependence and time course of closure of the inactivation gate. The mutations that cause hyperkalemic periodic paralysis are surprisingly on the intracellular end of hydrophobic transmembrane segments. The mechanism by which extracellular potassium may influence channel function at these positions is unclear. However, the positions of these mutations at the intracellular ends of S5 and S6 segments suggest that they may form part of the "receptor" for the inactivation gate as it closes. Mutations at the intracellular end of an S5 segment in the *Shaker* potassium channel inhibit channel inactivation in a manner that is interpreted as modification of the inactivation gate receptor in a ball-and-chain inactivation mechanism (128). Sequences at the intracellular end of segment IVS6 of the calcium channel are important for binding of phenylalkylamines, which are intracellular open channel blockers that enhance channel inactivation (253). These regions of the sodium channel may also surround the intracellular mouth of the transmembrane pore and serve as an intracellular receptor for closure of the inactivation gate. Structural changes at these sites are likely to affect inactivation by altering this receptor site structure.

XI. RETROSPECTIVE

Forty years after the Hodgkin and Huxley (122–125) and Hodgkin, Huxley, and Katz (126) papers, their impact on research on ion channels remains substantial. These papers established much of the conceptual basis and vocabulary of current research on the molecular properties of sodium channels. Their concepts of activation and inactivation gating particles have been given physical reality as molecular components of ion channels by a combination of molecular biological and biophysical experiments. It is rare that a series of papers remains cogent in its field of research 40 years later. This alone is a striking testimonial to the importance of this landmark work.

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