17 February 1984, Volume 223, Number 4637 SCIENCE

The Molecular Basis of Neuronal Excitability

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Summary. Neurons process and transmit information in the form of electrical signals. Their electrical excitability is due to the presence of voltage-sensitive ion channels in the neuronal plasma membrane. In recent years, the voltage-sensitive sodium channel of mammalian brain has become the first of these important neuronal components to be studied at the molecular level. This article describes the distribution of sodium channels among the functional compartments of the neuron and reviews work leading to the identification, purification, and characterization of this membrane glycoprotein.

Electrical excitability is among the most important and characteristic properties of neurons. Most vertebrate cells, including neurons, maintain large ionic gradients across their surface membranes such that the intracellular fluid contains a high concentration of potassium ions and low concentrations of sodium ions and calcium ions relative to the extracellular fluid. These large ion gradients are maintained by the action of energy-dependent ion pumps specific for Na^+ and K^+, or for Ca^{2+}. In addition, essentially all vertebrate cells maintain an internally negative membrane potential of the order of −60 millivolts since their surface membranes are specifically permeable to K^+ and this allows K^+ to leak out of cells faster than Na^+ and Ca^{2+} can leak in. Nerve cells are electrically excitable because of the presence, in their surface membranes, of voltage-sensitive ion channels that are selective for Na^+, K^+, or Ca^{2+}. One class of Na^+ channels and many classes of Ca^{2+} and K^+ channels have been described in neurons. These channels open and close as a function of membrane voltage allowing rapid movement of the appropriate ions down their concentration gradient so that ionic current passes into or out of the cell depolarizing or hyperpolarizing the membrane.

The electrical excitability of neurons is influenced by the nature of their ion channels, the density (number per unit area) of ion channels in their surface membranes, and the location of ion channels in the different functional compartments of the cell. Neurons can be divided into four morphological compartments, and each plays a different role in signal transmission (Fig. 1). Dendrites receive synaptic input from numerous presynaptic elements and respond with graded or, in some cases, propagated changes in membrane potential. The cell body or soma also receives synaptic inputs. It acts as a summing point for membrane potential changes occurring in various dendrites and on the soma itself. Depolarization of the cell membrane beyond a threshold value elicits one or a series of conducted action potentials which are initiated in the cell soma or the initial segment of the axon and are conducted down the axon to the nerve terminal. The action potential invades the nerve terminal causing depolarization, release of neurotransmitter into the synaptic cleft, and excitation of succeeding neurons in the pathway or of effector cells such as skeletal muscle. While Na^+, K^+, and Ca^{2+} channels each contribute in an essential way to signal processing and transmission in neurons, the role, mechanism of action, and molecular properties of the voltage-sensitive sodium channel are understood most completely. This article briefly reviews the physiological properties of sodium channels and then considers recent experiments that have begun to define the density and distribution of sodium channels in the different functional compartments of the nerve cell and the nature of the membrane macromolecules that comprise the sodium channel in neurons.

Physiological Properties of Sodium Channels

The ionic mechanisms underlying electrical excitability have been defined by the voltage clamp method (*1*, *2*). In this approach the voltage across the excitable membrane is controlled by means of a feedback amplifier circuit, and the ionic currents moving across the membrane in response to step changes in the membrane potential imposed by the experimenter are measured. The voltage clamp technique has been used to show that the initial rapid depolarization during an action potential in nerve axons results from rapid voltage-dependent increases in membrane permeability to sodium ions (*2*). Many different lines of evidence indicate that a selective transmembrane sodium channel is responsible for the rapid increase in sodium permeability during the action potential. Selective ion permeation is mediated by a hydrophilic pore containing a sodium-selective ion coordination site designated the ion selectivity filter (*3*). Ion conductance through the sodium channel is regulated or "gated" by two separate processes: (i) activation, which controls the rate and voltage-dependence of opening of the sodium channel after de-

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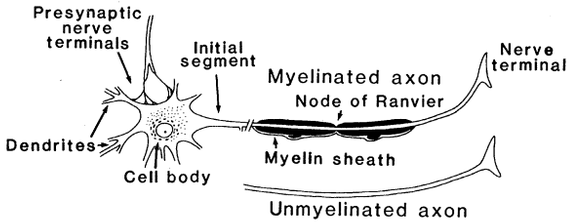

Fig. 1. Functional compartments of a "typical" vertebrate neuron. The size of the axon and nerve terminal is exaggerated relative to other structures and the distal portions of dendrites are not shown.

polarization and (ii) inactivation, which controls the rate and voltage-dependence of the subsequent closing of the sodium channel during a maintained depolarization (*2*). Estimates of the rate of sodium movement through an activated sodium channel derived from analysis of membrane current noise (*4*) or recordings of individual sodium channel currents (*5*) range from 8 to 18 picosiemens corresponding to more than 10^7 ions per second per channel at physiological temperature and Na^+ concentration. These rates approach those for diffusion through free solution and imply that the residence time of Na^+ protein ions in the channel is very short and that their interactions with the channel are weak.

Voltage clamp analysis has elucidated the three essential functional properties of sodium channels: voltage-dependent activation, voltage-dependent inactivation, and selective ion transport. However, an understanding of the molecular basis of neuronal excitability requires determination of the density and distribution of voltage-sensitive ion channels in neurons, identification of the membrane macromolecules that comprise the ionic channels, solubilization and purification of these channel components, and correlation of their structural features with the known functional properties of sodium channels.

Neurotoxins as Molecular Probes of Sodium Channels

Neurotoxins that bind with high affinity and specificity to voltage-sensitive sodium channels and modify their properties have provided the essential tools for identification and purification of sodium channels. Four different groups of neurotoxins that act at four different neurotoxin receptor sites on the sodium channel have been useful in these studies (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 (*6–8*).

Neurotoxin receptor site 2 binds several lipid-soluble toxins including grayanotoxin and the alkaloids veratridine, aconitine, and batrachotoxin (*8, 9*). The competitive interactions of these four toxins at neurotoxin receptor site 2 have been confirmed by direct measurements of specific binding of ^3H-labeled batrachotoxinin A 20α-benzoate to sodium channels (*10*). These toxins cause persistent activation of sodium channels at the resting membrane potential by blocking sodium channel inactivation and shifting the voltage dependence of the channel activation to more negative membrane potentials (*8*). Therefore, neurotoxin receptor site 2 is likely to be localized on a region of the sodium channel involved in voltage-dependent activation and inactivation.

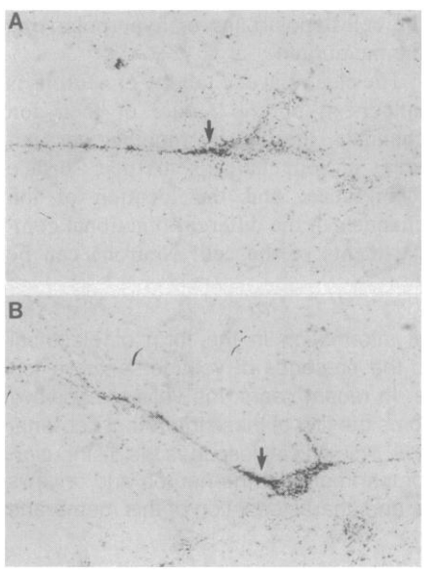

Fig. 2. Sodium channel distribution in cultured spinal neurons. Spinal cord neurons from embryonic rats were dissociated and maintained at low cell density in cell culture. After 3 weeks in vitro, cells were incubated with 1 n*M* ^{125}I-labeled scorpion toxin for 60 minutes to allow the toxin to bind to sodium channels, washed extensively to remove unbound toxin, and fixed in 2.5 percent glutaraldehyde. ^{125}I-Labeled scorpion toxin bound to sodium channels was detected by autoradiography (*22*). Two representative neurons are shown in bright-field microscopy to emphasize the autoradiographic grains. In each cell, the initial segment (arrows) of the largest neurite has a five- to sevenfold greater density of autoradiographic grains than the adjacent cell body. The autoradiographic labeling of sodium channels was prevented by competition with excess unlabeled scorpion toxin and by membrane depolarization. The nonneuronal cells that form a continuous monolayer on the substratum of these cultures were not labeled. The results show that sodium channels in neurons are labeled specifically.

Neurotoxin receptor site 3 binds polypeptide toxins purified from North African scorpion venoms or sea anemone nematocysts. These toxins slow or block sodium channel inactivation. They also enhance persistent activation of sodium channels by the lipid-soluble toxins acting at neurotoxin receptor site 2 (*8*). The affinity for binding of ^{125}I-labeled derivatives of the polypeptide toxins to neurotoxin receptor site 3 is reduced by depolarization. The voltage dependence of scorpion toxin binding is correlated with the voltage dependence of sodium channel activation (*11*). These data indicate that neurotoxin receptor site 3 is located on the part of the sodium channel that undergoes a conformational change during voltage-dependent channel activation leading to reduced affinity for scorpion toxin. Therefore, scorpion toxin and sea anemone toxin bind to voltage sensing or gating structures of sodium channels.

Neurotoxin receptor site 4 binds a new class of scorpion toxins that has also proved valuable in studies of sodium channels. Cahalan (*12*) showed that the venom of the American scorpion *Centruroides sculpturatus* modifies sodium channel activation rather than inactivation. Pure toxins from several American scorpions have a similar action (*13*). These toxins bind to a new receptor site on the sodium channel (*14*) and have been designated β scorpion toxins.

These several neurotoxins provide specific high affinity probes for distinct regions of the sodium channel structure. They have been used to detect and localize sodium channels in neuronal cells as well as to identify and purify the protein components of sodium channels that bind these toxins and to analyze their structural and functional properties.

Number and Distribution of Sodium Channels in Neurons

In vertebrate neurons, the density and distribution of sodium channels have been studied most completely in axons. Saxitoxin labeled with tritium by a specific isotopic exchange procedure has been used to determine the number of

high affinity saxitoxin binding sites, and therefore the number of sodium channels, in various nerves. These data, together with estimates of membrane surface area from microscopic studies, provide estimates of sodium channel density and localization. In the unmyelinated nerve fibers of the rabbit vagus, the density of sodium channels is 110 per square micrometer of membrane surface (*15*). Presumably these are distributed relatively uniformly along the fibers although no direct evidence is available on this point. To place these values in perspective, it is helpful to consider the portion of membrane surface occupied by the sodium channel. If the sodium channel is represented as a sphere with a mass of 320,000 daltons (see below), close-packed sodium channels would have a density of 14,800 per square micrometer. Thus, even in an axonal membrane specialized for conduction of action potentials, the sodium channel would occupy less than 1 percent of the surface area. Evidently the electrical excitability of neurons depends upon sparsely distributed but highly efficient ionic channels.

In unmyelinated axons, the action potential moves continuously down the axon with sodium channels in each succeeding segment of membrane being activated by the depolarization produced in the preceding segment. In contrast, vertebrate nerves that require high conduction velocity are myelinated and conduct action potentials discontinuously. In myelinated nerves, the axonal membrane is wrapped in many layers of myelin, an electrically inexcitable membrane produced by specialized glial cells. The axonal membrane is accessible to sodium ions in the extracellular fluid only at the nodes of Ranvier, interruptions of approximately 1 μm in length in the myelin sheath located at intervals of up to 1 mm. In saltatory conduction, the action potential "jumps" from node to node. The sodium ions entering the axon at one node must produce sufficient ionic current to depolarize the nodal membrane up to 1 mm away.

These large sodium currents flow across the membrane only at nodes of Ranvier, an indication of the high density of sodium channels at these sites. Comparison of the sodium current at the node with the ion conductance of a single sodium channel suggests a density of 2000 channels per square micrometer in the nodal membrane (*4*). Measurements of [^{3}H]saxitoxin binding suggest a substantially higher density of 12,000 per square micrometer, but these estimates required subtraction of the contribution of the unmyelinated fibers in the sciatic nerve, which is not known with certainty (*16*). Nevertheless, it is clear that nodes of Ranvier have a much higher density of sodium channels than other areas of the neuronal surface membrane.

Table 1. Neurotoxin receptor sites on the sodium channel.

Site	Neurotoxins	Physiological effect
1	Tetrodotoxin Saxitoxin	Inhibit ion transport
2	Veratridine Batrachotoxin Grayanotoxin Aconitine	Cause persistent activation
3	North African α scorpion toxins Sea anemone toxins	Slow inactivation
4	American β scorpion toxins	Enhance activation

The internodal region of myelinated axons is ensheathed in myelin and does not contribute to the ionic currents of the action potential. In order to test whether the underlying axonal membrane contains sodium channels, it is necessary to disrupt the myelin sheath. Several different kinds of experiments now indicate that there are few if any sodium channels located in internodal or perinodal regions of the myelinated axon. Homogenization of sciatic nerve to disrupt myelin does not reveal additional sodium channels as detected by high affinity binding of [^{3}H]saxitoxin (*16*). Osmotic shock of nodes of Ranvier under voltage clamp conditions exposes new axonal membrane in the perinodal region but does not increase the sodium current, an indication that few sodium channels are present in the perinodal membrane (*17*). Finally, antibodies to the purified sodium channel from electric eel electroplax (see below) have been used to localize sodium channels in eel motor nerve directly by immunocytochemical procedures (*18*). Tissue sections were incubated with antiserum to allow antibodies to bind to the sodium channel and then washed to remove unbound antibody. Bound antibody was then visualized with the peroxidase-antiperoxidase method. This immunocytochemical method also shows that sodium channels are highly concentrated in nodes of Ranvier and are not detectable in the perinodal region that is exposed by shearing of the nerves during sectioning.

The electrical properties of the cell bodies and dendrites of vertebrate neurons are complex and incompletely understood. Sodium-dependent action potentials are detected in neuronal cell bodies but are less important in dendrites (*19*). The density of sodium channels in cell bodies and dendrites of vertebrate neurons in situ has not been measured. However, measurements of [^{3}H]saxitoxin binding to the cell bodies of neural tumor cells maintained in cell culture indicate a density of sodium channels of 51 to 75 μm^{-2} (*20*). These values are in the same range as those for unmyelinated axons and are much lower than those for nodes of Ranvier.

Studies of excitation of spinal neurons show that in motor neurons and many interneurons the threshold for action potential generation is lower at the initial segment of the axon than elsewhere on the cell soma (*21*). These studies indicate a discontinuity of membrane electrical properties at the junction of cell body and axon. Direct determination of the distribution of sodium channels in rat spinal neurons maintained in cell culture has been made with ^{125}I-labeled scorpion toxin. The cells were allowed to bind labeled scorpion toxin to neurotoxin receptor site 3 on sodium channels. The bound toxin was covalently attached to its receptor site by fixation with glutaraldehyde. The bound toxin molecules were then located by autoradiography. These measurements of specifically bound scorpion toxin reveal inhomogeneity in channel distribution (*22*). In approximately 40 percent of cultured spinal neurons, the density of sodium channels in the initial segments of one or more major neuronal processes is sevenfold higher than in the adjacent cell body (Fig. 2). Evidently, many spinal neurons maintain a high density of sodium channels in the initial segments of their axons in cell culture in the absence of the three-dimensional structure of the spinal cord. The higher channel density reduces threshold for excitation by allowing generation of a propagated action potential when a smaller fraction of the channels is activated. The high probability of initiation of action potentials at the initial segment is a determinant of the integrative properties of these neurons.

Table 2. Composite view of sodium channel distribution in a "typical" mammalian neuron. Fragmentary evidence from experiments in several different systems (see text) permits an estimation of the probable sodium channel density and distribution in a "typical" neuron.

Cell compartment	Sodium channel density (μm^{-2})	Method
Cell body	50 to 75	Saxitoxin binding
Axon initial segment	350 to 500*	Scorpion toxin autoradiography*
Unmyelinated axon	110	Saxitoxin binding
Myelinated axon node of Ranvier	2000 to 12000	Electrophysiology Saxitoxin binding
Internode	< 25	Electrophysiology Saxitoxin binding Immunocytochemistry
Nerve terminals	20 to 75	Saxitoxin binding Scorpion toxin binding

*These values were derived by multiplying the sodium channel density in the cell bodies of cultured neural cell lines [50 to 75 μm^{-2} (*20*)] by the ratio of channel density on neurite initial segments to that on cell bodies of cultured spinal neurons [7.0 (*22*)].

Action potentials propagated down axons depolarize nerve terminals causing activation of calcium channels and release of neurotransmitters. The small size of nerve terminals has prevented electrophysiological examination by intracellular recording. Investigation of the properties of pinched off nerve terminals (synaptosomes) isolated from homogenized mammalian brain by differential and density gradient centrifugation has provided evidence that there is a substantial number of sodium channels in nerve terminals. Activation of sodium channels with veratridine, batrachotoxin, sea anemone toxin, or scorpion toxin elicits calcium-dependent neurotransmitter release from synaptosomes (*23*) and increases their sodium permeability (*24*). Purified synaptosomal fractions contain a substantial complement of sodium channels as assessed by specific binding of ^{125}I-labeled scorpion toxin (*25*) or [^{3}H]saxitoxin (*26*). The density of sodium channels derived from measurements of saxitoxin and scorpion toxin binding is in the range of 20 to 75 μm^{-2}, somewhat lower than in unmyelinated nerve membrane and in the cell bodies of neural cells in culture.

Table 2 summarizes the distribution of sodium channels in a "typical" neuron. While the available information remains fragmentary, it now appears that sodium channels are present at a density in the range of 50 to 110 μm^{-2} in cell bodies and unmyelinated axons while nerve terminals have somewhat lower densities. Higher densities are observed in specific localizations in nodes of Ranvier and in the initial segments of the axons of at least some neurons. The density of sodium channels in the internodal membrane of myelinated axons is relatively low, and these channels may be absent in most dendrites. This distribution plays a critical role in determining the signal processing properties of neurons. Membrane potential changes in the dendrites are graded and represent the sum of the contributions of multiple synaptic inputs. The summed potentials reach the cell body and are modulated by additional synaptic inputs to the soma. There are enough sodium channels in the cell body for conducted action potentials to be initiated there. However, in some neurons, a high density of sodium channels at the axon initial segment reduces the threshold for action potential generation at that site, so that the initial segment acts as a summation point for graded potentials from both dendrites and soma. Action potentials propagate down axons by slow continuous conduction in unmyelinated nerves or by more rapid discontinuous (saltatory) conduction in myelinated nerves depending on the distribution of sodium channels in the axonal membrane. Since the localization of sodium channels is an important determinant of the electrical excitability and integrative properties of neurons, it is important to examine channel distribution in identified classes of central and peripheral neurons and attempt to relate these observations to the physiological properties of the nerve cells. It seems likely that there will be many individual variations on the "typical" neuron illustrated in Table 2.

Identification of Protein Components of Sodium Channels in Neurons

Measurements of the distribution and density of sodium channels indicate that, with the exception of the very small amount of specialized membrane at the node of Ranvier, sodium channels are a minor component of excitable membranes. These results emphasize the need for highly specific probes to identify the macromolecules that comprise the sodium channel. The neurotoxins that bind to sodium channels with high affinity and specificity have provided the tools needed in such experiments. 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 α scorpion toxin from *Leiurus quinquestriatus*. The photoreactive toxin derivative is allowed to bind specifically to sodium channels in the dark. Irradiation with ultraviolet light then chemically activates the arylazide group, which covalently reacts with the scorpion toxin receptor site on the sodium channel. Analysis of covalently labeled synaptosomes by polyacrylamide gel electrophoresis under denaturing conditions in sodium dodecyl sulfate (SDS) to separate synaptosomal proteins by size reveals specific covalent labeling of two polypeptides that were subsequently designated the α and β1 subunits of the sodium channel (Fig. 3, lane 1) (*27*). These proteins, as assessed by polyacrylamide gel electrophoresis in SDS, have molecular sizes of 270,000 and 39,000 daltons, respectively.

The covalent labeling of these two polypeptides in synaptosomes was shown to be specific by inhibition by competition with excess unlabeled scorpion toxin (Fig. 3, lanes 3 and 4) or by blockade of voltage-dependent binding of scorpion toxin by membrane depolarization (Fig. 3, lanes 5 and 6) (*27*). The α subunit of the sodium channel could also be covalently labeled with azidonitrobenzoyl scorpion toxin in electrically excitable neuroblastoma cells. In contrast, in mutant neuroblastoma cells that are neurotoxin-resistant and lack functional voltage-sensitive sodium channels, the 270,000-dalton polypeptide corresponding to the α subunit is not present (*27, 28*). These data provide additional evidence for the specificity of photoaffinity labeling.

The β scorpion toxins derived from American scorpion venoms have been used to label neurotoxin receptor site 4 on the sodium channel (*29*). Toxin from *Tityus serrulatus* was covalently attached to its receptor site by cross-linking with disuccinimidyl suberate. A single polypeptide of 270,000 daltons was labeled in rat brain synaptosomes. Thus, the receptor site for the β scorpion toxins is located on or near the α subunit of the sodium channel, as previously found for the α scorpion toxins acting at neurotoxin receptor site 3.

Molecular Size of the Sodium Channel

The first indications of the molecular size of the neuronal sodium channel in situ were derived from radiation inactivation studies (*30*). In these experiments, membrane preparations from pig brain were irradiated with x-rays, and the decrease in the number of functional tetrodotoxin binding sites was measured as a function of radiation dose. From these data, the size of the membrane target can be determined since larger targets are more likely to be hit and are therefore inactivated at a lower radiation dose. Applying target theory, Levinson and Ellory concluded that a structure of 230,000 daltons was required for toxin binding. These experiments have recently been repeated by Barhanin *et al.* (*29*) who compared the target size of the sodium channel assessed by either tetrodotoxin binding or *Tityus serrulatus* toxin γ binding. In each case, the target size was approximately 270,000 daltons, in reasonable agreement with the earlier work. This size estimate might correspond to the molecular size of the entire sodium channel or to that of a protein subunit that is essential for binding these neurotoxins.

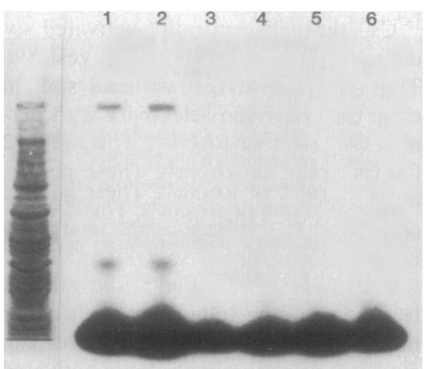

Fig. 3. Identification of the protein components of sodium channels by covalent labeling with photoreactive scorpion toxin. Intact synaptosomes were incubated with 2 n*M* azidonitrobenzoyl scorpion toxin labeled with ^{125}I to allow the toxin to bind to neurotoxin receptor site 3 on the sodium channel; the synaptosomes were then irradiated with ultraviolet light to activate the photoreactive arylazide. Binding reaction conditions were as follows: (lane 1) 1 μ*M* tetrodotoxin; (lane 2) 1 μ*M* tetrodotoxin and 1 μ*M* batrachotoxin; (lane 3) 1 μ*M* tetrodotoxin and 200 n*M* unlabeled scorpion toxin; (lane 4) 1 μ*M* tetrodotoxin, 1 μ*M* batrachotoxin, and 200 n*M* unlabeled scorpion toxin; (lane 5) 135 m*M* KCl, 1 μ*M* tetrodotoxin, and 1 μ*M* batrachotoxin; and (lane 6) 135 m*M* KCl, 1μ*M* tetrodotoxin, 1 μ*M* batrachotoxin, and 200 n*M* unlabeled scorpion toxin. Samples were analyzed by SDS gel electrophoresis and covalently bound ^{125}I-labeled scorpion toxin was detected by autoradiography. A Coomassie blue–stained gel lane is shown on the left.

The molecular size of the intact sodium channel protein has been measured by hydrodynamic studies of the detergent-solubilized channel. The saxitoxin and tetrodotoxin binding component of sodium channels was first solubilized with retention of high affinity and specificity of toxin binding from garfish olfactory nerve by treatment with nonionic detergents (*31*). Similar techniques have now been applied to sodium channels in mammalian brain (*26*).

In contrast to the ease of solubilization of the sodium channel with retention of saxitoxin and tetrodotoxin binding activity at neurotoxin receptor site 1, both neurotoxin receptor site 2 and neurotoxin receptor site 3 (*26*) lose their high affinity neurotoxin binding activity on solubilization. The molecular size of the solubilized sodium channel from rat brain has been estimated by hydrodynamic studies to be 601,000 daltons (*32*). Since the detergent-channel complex contains 0.9 g of Triton X-100 and phosphatidylcholine per gram of protein, the size of the sodium channel protein solubilized from rat brain is 316,000 daltons (*32*). This represents the size of the entire sodium channel as solubilized in detergents and corresponds to a complex of three nonidentical protein subunits as described below. If the channel protein is spherical in shape, the diameter indicated by these results is 118 Å. Thus, the channel protein is much larger than the postulated transmembrane pore through which Na^+ moves which is proposed to be 3 by 5 Å at its narrowest point, the ion selectivity filter (*3*).

Protein Subunits of the Purified Sodium Channel from Mammalian Brain

The ability to solubilize the sodium channel from brain membranes in a well-defined monomeric form with retention of binding activity for saxitoxin and tetrodotoxin has allowed purification by a sequence of conventional protein separation procedures (*33*). The current purification scheme developed with my colleagues consists of anion exchange chromatography on DEAE-Sephadex, adsorption chromatography on hydroxylapatite gel, affinity chromatography on wheat germ agglutinin covalently attached to Sepharose 4B, and velocity sedimentation through sucrose gradients (*34*). The purified sodium channel preparation binds 0.9 mole of saxitoxin per mole of sodium channel of 316,000 daltons. If the sodium channel binds only one saxitoxin molecule, these data indicate that at least 90 percent of the protein in the purified preparation must be associated with the sodium channel.

The protein subunits of the sodium channel have been analyzed by denaturation of the protein at 100°C in the presence of SDS and β-mercaptoethanol with subsequent separation according to molecular size by electrophoresis in polyacrylamide gels; two protein bands with molecular sizes of 260,000 and 38,000 daltons can be resolved from the purified sodium channel with this technique (Fig. 4A) (*33, 34*). These two bands, which we designate α and β, contain more than 90 percent of the protein in the purified preparation and comigrate precisely with the saxitoxin binding activity of the sodium channel during velocity sedimentation on sucrose gradients (*34*). Thus, the sodium channel isolated from rat brain appears to be 90 percent pure by both chemical and functional criteria and consists of at least two classes of subunits.

Often the β protein band appears as a closely spaced doublet, suggesting that it might contain two different polypeptides (*34*). Analysis of the effect of reduction of protein disulfide bonds on the migration of the α and β protein bands has shown that the β protein band corresponds to two nonidentical subunits of similar molecular size (*34*). Purified sodium channels that are denatured in SDS without reduction of disulfide bonds can

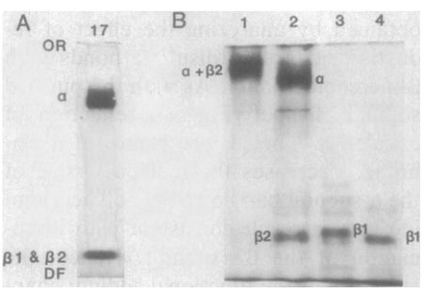

Fig. 4. Subunit composition of the purified sodium channel. (A) The purified sodium channel from fraction 17 of a sucrose gradient sedimentation was denatured by incubation at 100°C in SDS and β-mercaptoethanol, and its polypeptide subunits were separated according to size by SDS gel electrophoresis. The separated α and β subunits were then visualized by a sensitive silver staining procedure. Ninety percent of the silver stain in the gel was on the α and β subunit bands, indicating that the sodium channel is 90 percent pure (*34*). (B) Purified sodium channels were denatured by incubation at 100°C in SDS without β-mercaptoethanol and separated according to size by gel filtration (Sepharose 4B-CL). These two fractions were analyzed by SDS gel electrophoresis and visualized by silver stain as described (*34*). (Lane 1) High molecular weight fraction without reduction; (lane 2) high molecular weight fraction with reduction; (lane 3) low molecular weight fraction with reduction; and (lane 4) low molecular weight fraction without reduction.

be separated into two fractions by gel filtration (*34*). Analysis of the high-molecular weight fraction by SDS gel electrophoresis reveals only a single protein band of 300,000 daltons (Fig. 4B, lane 1). Reduction of disulfide bonds with β-mercaptoethanol splits this protein into two subunits, the α subunit (260,000 daltons) and the β2 subunit (37,000 daltons) (Fig. 4B, lane 2). In contrast, the low molecular weight fraction contains only a single polypeptide subunit, β1 (39,000 daltons), when analyzed with or without reduction (Fig. 4B, lanes 3 and 4). These results show that the purified sodium channel from rat brain consists of three protein subunits: α, 260,000 daltons; β1, 39,000 daltons, and β2, 37,000 daltons (Table 3). These subunits are associated in a stoichiometric complex of 316,000 daltons (*32*) containing the saxitoxin receptor site of the sodium channel.

Photoaffinity labeling experiments in which the protein components of sodium channels are covalently labeled and analyzed in situ in synaptosomes have provided evidence that a similar complex of α, β1, and β2 is present in intact synaptosomal membranes where the sodium channel is fully functional. The α and β1 subunits are covalently labeled by scorpion toxin and therefore must be located at or near neurotoxin receptor site 3 on the channel (Fig. 3) (*27, 34*). The β2 subunit is not covalently labeled, but indirect evidence for its presence was obtained by analyzing the effect of reduction of protein disulfide bonds with β-mercaptoethanol. As with the purified sodium channel (Fig. 4), reduction of covalently labeled synaptosomal membranes decreases the molecular size of the α subunit band on SDS polyacrylamide gels, a result consistent with dissociation of the β2 subunit. Hence, the structure of the functional sodium channel in synaptosomes is similar or identical to that described for the purified channel. However, these experiments do not exclude the possibility that alteration of channel structure by proteolysis or other means may have occurred during homogenization of the brain and isolation of the synaptosomal fractions, despite precautions taken against such effects.

Table 3. Subunit composition of the sodium channel purified from rat brain.

Protein	Molecular size (daltons)	Probable stoichiometry
Native Na	316,000	
α subunit	260,000	1.0
β1 subunit	39,000	1.0
β2 subunit	37,000	1.0

Reconstitution of Sodium Channel Function from Purified Components

The purified sodium channel from rat brain binds [^{3}H]saxitoxin and tetrodotoxin with the same affinity as the native sodium channel and therefore contains neurotoxin receptor site 1 of the sodium channel in an active form (*34*). The purified channel also contains the α and β1 subunits that were identified as components of neurotoxin receptor site 3 by photoaffinity labeling with scorpion toxin (*27*), although after solubilization the binding activity for scorpion toxin is lost (*34*). However, the purified channel does not have binding activity for neurotoxins at receptor site 2 and cannot transport sodium in the detergent-solubilized state. Reconstitution of these sodium channel functions from purified components is the only rigorous proof that the proteins identified and purified on the basis of their neurotoxin binding activity are indeed sufficient to form a functional voltage-sensitive ion channel. In addition, successful reconstitution will provide a valuable experimental preparation for biochemical analysis of the structure and function of sodium channels.

Several groups of investigators have successfully restored aspects of sodium channel function from detergent-solubilized brain membranes and have shown that detergent solubilization does not irreversibly destroy channel function (*35*). More recently, sodium channel ion transport has been successfully reconstituted from sodium channels substantially purified from rat brain and skeletal muscle (*36*). We have now applied these methods to essentially homogeneous preparations of sodium channels from rat brain (*37*). Purified sodium channels in Triton X-100 solution are supplemented with phosphatidylcholine dispersed in Triton X-100, and the detergent is removed by adsorption to polystyrene beads. As the detergent is removed, phosphatidylcholine vesicles with a mean diameter of 1800 Å are formed containing an average of 0.75 to 2 sodium channels per vesicle. The functional activities of the sodium channel can then be assessed in neurotoxin binding and ion flux experiments.

The time course of $^{22}Na^+$ influx into phosphatidylcholine vesicles containing purified sodium channels is illustrated in Fig. 5A. The vesicle preparation was incubated for 2 minutes with veratridine to activate sodium channels and then diluted into medium containing $^{22}Na^+$ to initiate influx into vesicles. Influx into vesicles under control conditions was slow (Fig. 5A). Incubation with veratridine increased the initial rate of influx 10- to 15-fold. When tetrodotoxin was present in both the intravesicular and extravesicular phases, the veratridine-dependent increase in initial rate of $^{22}Na^+$ influx was nearly completely blocked (Fig. 5A). Half-maximal activation was observed with 28 μ*M* veratridine and half-maximal inhibition with 14 n*M* tetrodotoxin in close agreement with the corresponding values for the action of these toxins on native sodium channels. These results show that the purified sodium channel regains the ability to mediate neurotoxin-stimulated ion flux after incorporation into phosphatidylcholine vesicles. Evidently, the purified channel retains neurotoxin receptor site 2 and the ion-conducting pore of the sodium channel.

Ion transport by neurotoxin-activated sodium channels in neural membranes is selective although the rate of transport of large cations such as Rb^+ and Cs^+ relative to the rate of transport of Na^+ is significantly greater than when channels are activated by membrane depolarization (*38*). Figure 5B illustrates the initial rate of influx of $^{22}Na^+$, $^{86}Rb^+$, and $^{137}Cs^+$ through veratridine-activated sodium channels in reconstituted vesicles. The purified and reconstituted sodium channel retains ion selectivity with permeability ratios of 0.25 for Rb^+ and 0.12 for Cs^+ relative to Na^+. These permeability ratios compare favorably to those of native sodium channels activated by neurotoxins.

The above results (Fig. 5) show that at least some of the sodium channels in our most highly purified preparations can mediate selective neurotoxin-activated ion transport after incorporation into phospholipid vesicles. My colleagues and I have attempted to estimate how many of the reconstituted sodium channels contribute to our ion flux measurements. First, the ion transport rates in purified and reconstituted sodium channel preparations were compared to those of veratridine-activated sodium channels in neuroblastoma cells and synaptosomes (*37*). This comparison shows that the transport rate measured in ions per minute per saxitoxin receptor site is 33 to 70 percent of that in native membranes, suggesting that at least 33 to 70 percent of the reconstituted sodium channels are active. Second, we have compared the proportion of vesicles that contain sodium channels to the proportion of vesicles whose internal volume is accessible to

veratridine-activated sodium channels (*37*). If sodium channels are distributed among vesicles according to a Poisson distribution, this comparison leads to a range of 30 to 70 percent for the fraction of active channels, depending on whether active vesicles containing more than one channel are assumed to have one active channel or all active channels. Both of these estimates indicate that a minimum of 30 percent of the reconstituted sodium channels are active. Since the sodium channel preparation is 90 percent pure and no single contaminant comprises as much as 2 percent of the protein, we conclude that the purified complex of α, β1, and β2 is sufficient to mediate selective neurotoxin-activated ion flux.

While sodium channels reconstituted into phosphatidylcholine vesicles can transport sodium, these channels do not bind α scorpion toxin at neurotoxin receptor site 3. In contrast, if purified sodium channels are incorporated into vesicles composed of a mixture of phosphatidylcholine and brain lipids, scorpion toxin binding is recovered. The toxin binding reaction is of high affinity (K_D = 57 n*M*) and a mean of 0.76 ± 0.08 mole of scorpion toxin is bound per mole of purified sodium channel (*37*). The brain lipid fraction was purified by extraction with a mixture of chloroform and methanol followed by silicic acid chromatography. It was found to be protein-free by gel electrophoresis and was sensitive to silver staining. Therefore, we conclude that brain lipids are essential to restore the scorpion toxin receptor site to the same functional state as in native membranes. Since the affinity for scorpion toxin binding to synaptosomal sodium channels is dependent on the functional state of the sodium channel (*11, 24, 25*), components of the brain lipid mixture may also be required for other functional activities of the channel. Since the scorpion toxin binding in reconstituted vesicles containing brain lipid is not voltage-dependent (*37*), the lipid environment provided by the mixture of phosphatidylcholine and whole brain lipid may not be optimal for channel function. Further analysis of the lipid environment necessary for recovery of channel function may reveal requirements for specific membrane lipids for voltage sensitivity.

The results of these reconstitution experiments show that the purified sodium channel preparation from rat brain consisting of a stoichiometric complex of the α, β1, and β2 subunits is sufficient to mediate most of the functions of the sodium channel that can be measured biochemically. These include neurotoxin binding and action at neurotoxin receptor sites 1 through 3 and selective neurotoxin-activated ion flux. However, in excitable membranes sodium channels are normally activated and inactivated by changes in membrane potential. Purified and reconstituted channels have not yet been tested for their ability to activate and inactivate on depolarization. Assessment of this aspect of channel function awaits application of methods to record electrically from purified channels in large reconstituted vesicles or planar bilayers. Such experiments should provide the final test for the functional integrity of the purified and reconstituted channels.

Posttranslational Modification of the Neuronal Sodium Channel

Membrane proteins are often covalently modified after biosynthesis of their polypeptide chains. Glycosylation at asparagine residues and proteolytic cleavage accompany insertion into membranes and transport to the cell surface. Phosphorylation by specific protein kinase is a common mediator of cellular regulatory processes. The sodium channel from rat brain is specifically adsorbed and eluted from chromatographic columns containing immobilized wheat germ agglutinin, a carbohydrate-binding protein with specificity for *N*-acetylglucosamine and sialic acid residues in glycoproteins (*33*). These results indicate that the sodium channel is a glycoprotein. Further chemical analyses have shown that all three of the subunits of the sodium channel from rat brain are glycosylated. At present, there is no evidence for an essential role of the carbohydrate moieties in the function of sodium channels in neuronal surface membranes. However, blockade of protein glycosylation with the specific inhibitor tunicamycin reduces the level of functional sodium channels in cultured neuroblastoma cells to less than 20 percent of control levels (*39*). After glycosylation is blocked, the existing sodium channels are lost with a half-time of 24 hours, and protein synthesis is required to restore the normal level of functional sodium channels after removal of tunicamycin (*39*). These results indicate that glycosylation is required for the normal biosynthesis, membrane insertion, and maintenance of functional sodium channels in neural cells.

Protein phosphorylation is one of the most important mechanisms of cellular regulation (*40*) and has been implicated in the long-term regulation of neuronal excitability (*41*). The rat brain sodium channel is also modified by phosphorylation and dephosphorylation. The purified sodium channel is phosphorylated on the α subunit by cyclic adenosine monophosphate (cyclic AMP) dependent protein kinase added in vitro (*42*). Kinetic analysis of the rate and extent of phosphorylation indicates that the sodium channel is a physiological substrate for cyclic AMP–dependent protein ki-

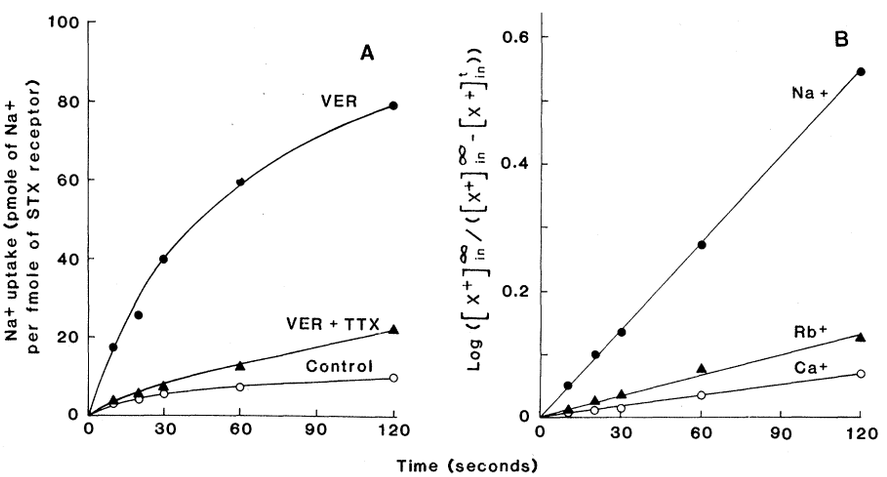

Fig. 5. Rate and ion selectivity of veratridine-activated ion flux by the purified and reconstituted sodium channel. (A) Phosphatidylcholine vesicles containing purified sodium channels were incubated for 2 minutes at 36°C in control medium (○) or with 100 μ*M* veratridine (●), and the initial rate of $^{22}Na^+$ influx was measured in the same medium as described (*36, 37*). An identical sample of vesicles was prepared with 1 μ*M* tetrodotoxin inside, incubated for 2 minutes at 36°C with 100 μ*M* veratridine and 1 μ*M* tetrodotoxin, and the initial rate of $^{22}Na^+$ influx was measured (▲). (B) Veratridine-activated ion flux was measured as in (A) and the control rate of influx was subtracted for ^{22}Na, ^{86}Rb, and ^{137}Cs. The data are presented as a linear first-order rate plot in which the slope is proportional to ion permeability. Symbols: *STX*, saxitoxin; *VER*, veratridine; and *TTX*, tetrodotoxin.

nase. More recently, the development of antiserum to the purified sodium channel has allowed measurement of phosphorylation of the α subunit of the sodium channel in intact synaptic nerve ending particles (synaptosomes). We found that addition of the 8-bromo derivative of cyclic AMP causes complete phosphorylation of the sodium channel in synaptosomes in 15 seconds (*42*). Phosphorylation occurred at the same sites that were phosphorylated on the α subunit of the purified sodium channel (*42*). These results show that the sodium channel is phosphorylated in situ in nerve terminals when intracellular cyclic AMP increases. Phosphorylation is associated with a decrease in the maximum rate of toxin-activated $^{22}Na^+$ influx and possibly a small increase in the concentration of neurotoxins required for half-maximal activation (*42*). These results indicate that phosphorylation reduces persistent activation of sodium channels by neurotoxins. Electrophysiological experiments are required to determine whether this reflects either a reduced probability of sodium channel activation or an increased probability of inactivation by membrane depolarization. Small changes in the voltage dependence of either of these channel functions would be expected to have important effects on the threshold and frequency of action potential generation in neurons and on the extent of impulse-induced release of neurotransmitters from nerve terminals. Such changes may serve to modulate the response of neurons to their synaptic inputs on a long-term basis.

Sodium Channels in Skeletal Muscle and Electric Organ

Action potentials in skeletal muscle and in the electroplax of the electric eel *Electrophorus electricus* are also sodium dependent and are mediated in part by voltage-sensitive sodium channels with properties similar to those in nerve. Concomitant with the work described here, similar studies on sodium channels in these tissues are being carried out. Neurotoxin binding experiments with [^{3}H]saxitoxin or tetrodotoxin have shown that the density of sodium channels is 200 to 380 μm^{-2} in muscle surface membrane (*7*) and 200 to 500 μm^{-2} in the electric organ (*43*), which is derived from muscle during embryogenesis. The size of the tetrodotoxin-binding component of the sodium channel from electroplax determined by x-ray target size analysis is 230,000 daltons (*30*) and the purified protein consists of a single glycoprotein subunit with a molecular size of 260,000 daltons (*44*). Presumably this polypeptide is analogous to the α subunit of the sodium channel from rat brain. The purified tetrodotoxin binding component from electric eel does not appear to have subunits analogous to the β1 and β2 subunits of the sodium channel purified from rat brain. However, since functional reconstitution of this channel preparation has not yet been achieved, it remains possible that additional subunits will be required for full activity.

The size of the detergent-solubilized sodium channel from rat skeletal muscle is 315,000 daltons (*45*). The purified muscle sodium channel consists of a large glycoprotein subunit of uncertain molecular size (150,000 to 250,000 daltons), a subunit of 47,000 daltons, and a pair of subunits of approximately 38,000 daltons (*45*). This purified preparation has been incorporated into phosphatidylcholine vesicles with recovery of ion transport function (*45*). The large subunit and the two subunits of approximately 38,000 daltons are likely to be analogous to the α, β1, and β2 subunits of the rat brain sodium channel. Thus, the sodium channel proteins from brain, skeletal muscle, and electroplax consist primarily of a large glycoprotein subunit of approximately 250,000 daltons. In addition, the sodium channel from mammalian tissues contains smaller polypeptides that may be essential for sodium channel function.

Conclusion

In the past several years, new experimental approaches have allowed important new advances in understanding the molecular basis of electrical excitability, a critical problem in neurobiology. This article has focused on insights derived from the use of specific neurotoxins as molecular probes to define the density and distribution of voltage-sensitive sodium channels in neurons and to identify, purify, and characterize the molecular components of sodium channels in neuronal surface membranes. This general approach has shown that sodium channels are sparsely distributed in excitable membranes and has provided estimates of channel densities in different neuronal compartments. The uneven distribution of sodium channels observed in the specialized regions of neurons confirm the suggestions from electrophysiological studies that regulation of the density of sodium channels in neuronal membranes is an important determinant of the different functional roles of the dendrites, cell soma, axon, and nerve endings in signal transmission. Variation of sodium channel distribution among individual neurons may play an important role in their signal transmission.

The use of specific neurotoxin probes has also provided the first information on the biochemical nature of a voltage-sensitive ion channel and allowed the first purification and functional reconstitution of a protein involved in signal processing and transmission in neurons. The polypeptide components of sodium channels in nerve, skeletal muscle, and electroplax have been identified, highly purified, and shown to be large membrane glycoproteins. Functional reconstitution of neurotoxin binding and ion transport activity has been achieved from purified components. The general structural features and subunit interactions of the neuronal sodium channel have been defined. These experiments represent the first steps toward relating the structural features of ion channels with their functional properties. Of particular interest is determination of the structural features that confer the extreme voltage sensitivity on channel function and allow rapid and selective ion transport. Higher resolution structural analysis and determination of the functional properties of sodium channels having discrete changes in biochemical structure will be the next essential steps toward this goal. The availability of highly purified preparations of sodium channels which retain functional activity opens the way for additional progress in understanding the molecular basis of neuronal excitability.

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RESEARCH ARTICLE

Malignant Activation of a K-*ras* Oncogene in Lung Carcinoma but Not in Normal Tissue of the Same Patient

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Oncogenes capable of inducing malignant transformation on transfection of NIH/3T3 cells have been found in various human tumor cell lines, as well as in unmanipulated solid tumors (*1*). Most of these transforming genes belong to the *ras* gene family (*2–5*), which acquires malignant properties on recombination with retroviral sequences (*6, 7*). Three human *ras* oncogenes, H-*ras* (*4, 8, 9*), K-*ras* (*10–12*), and N-*ras* (*13–15*), have been characterized. Although the *ras* genes have different genetic structures, all of them code for proteins of 189 amino acid residues, generically designated p21 (*16*).

Molecular characterization of the human H-*ras* and K-*ras* loci indicates that these genes acquire malignant properties by single point mutations that affect the incorporation of the 12th or 61st amino acid residue of their respective p21 proteins (*10, 12, 17–23*). Mutations within codon 12 of H-*ras* alter the sequence CCGG (C, cytosine; G, guanine), which is specifically recognized by certain restriction endonucleases, thus providing a simple biochemical assay for detection of transforming H-*ras* genes. However, the potential use of these findings is hampered by the infrequent activation of the H-*ras* locus in human tumors (*24, 25*). In contrast, K-*ras* oncogenes have been detected in various human neoplasias (*1*). We have tested 96 human tumors or tumor cell lines for the ability of their DNA's to transform NIH/3T3 cells. Of these, only T24 bladder carcinoma cells had a mutated H-*ras* locus, whereas transforming K-*ras* genes have been identified in eight different carcinomas and sarcomas, including three unmanipulated solid tumors (*25*).

We now report that two of the six possible single point mutations that can activate the human K-*ras* locus by altering the coding properties of its 12th codon create restriction enzyme polymorphisms. These polymorphisms have been utilized to develop biochemical assays that discriminate between normal and transforming K-*ras* genes. We have used such assays to determine the mechanism of activation of K-*ras* oncogenes in A1698 bladder carcinoma and A2182 lung carcinoma human tumor cell lines (*25*). Moreover, we have established that a single point mutation responsible for the malignant activation of a K-*ras* oncogene is present in tumor but not in normal tissue of a patient with a squamous cell lung carcinoma. These results demonstrate an association between activation of *ras* oncogenes by specific mutational mechanisms and the development of certain human cancers.

Polymorphisms in K-*ras* Oncogenes

Although the clinical manifestation of a tumor is not strictly correlated with the activation of a particular member of the *ras* gene family, K-*ras* oncogenes have been preferentially identified in human tumors. Thus, molecular characterization of K-*ras* might help to reveal the role of transforming genes in human carcinogenesis. Because of the complexity of the human K-*ras* locus (*10–12*) we limited our cloning efforts to DNA segments containing exon sequences. We subcloned domains of v-*kis*, the *onc* gene of the Kirsten strain of murine sarcoma

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