Structure and Function of Voltage-Sensitive Ion Channels

WILLIAM A. CATTERALL

Voltage-sensitive ion channels mediate action potentials in electrically excitable cells and play important roles in signal transduction in other cell types. In the past several years, their protein components have been identified, isolated, and restored to functional form in the purified state. Na^+ and Ca^{2+} channels consist of a principal transmembrane subunit, which forms the ion-conducting pore and is expressed with a variable number of associated subunits in different cell types. The principal subunits of voltage-sensitive Na^+, Ca^{2+}, and K^+ channels are homologous members of a gene family. Models relating the primary structures of these principal subunits to their functional properties have been proposed, and experimental results have begun to define a functional map of these proteins. Coordinated application of biochemical, biophysical, and molecular genetic methods should lead to a clear understanding of the molecular basis of electrical excitability.

THE ION CHANNELS INVOLVED IN THE GENERATION OF electrical signals can be grouped into two classes. Ligand-gated ion channels such as the nicotinic acetylcholine receptor, γ-aminobutyric acid (GABA) receptor, and glycine receptor mediate local increases in ion conductance at chemical synapses and thereby depolarize or hyperpolarize the subsynaptic area of the cell. In contrast, voltage-sensitive ion channels mediate rapid, voltage-gated changes in ion permeability during action potentials in excitable cells and also modulate membrane potential and ion permeability in many inexcitable cells (*1*). The voltage-sensitive ion channels are characterized by single channel conductance rates in the range of 10^7 per second, high ion selectivity, and steep voltage sensitivity of their ion conductance activity.

In most excitable cells, the action potential consists of three phases. A rapid increase in Na^+ permeability mediated by voltage-sensitive Na^+ channels causes rapid depolarization during the initial phase of the action potential. The cell remains depolarized during the plateau phase of the action potential because of the inward movement of Ca^{2+} ions through voltage-sensitive Ca^{2+} channels. The Ca^{2+} entering cells during this phase of the action potential serves as the primary intracellular second messenger for the electrical signals generated in the plasma membrane, and initiates excitation-contraction coupling, excitation-secretion coupling, and multiple Ca^{2+} activated biochemical processes. The action potential is terminated by activation of voltage-sensitive K^+ channels that mediate outward movement of K^+ ions, which repolarizes the cell. Voltage-sensitive K^+ channels also set the resting membrane potential of the cell and modulate action potential frequency and threshold.

The ion conductance activity of voltage-sensitive ion channels is controlled on the millisecond time scale by two experimentally separable processes: (i) voltage-dependent activation, which controls the time- and voltage-dependence of ion channel opening in response to changes in membrane potential, and (ii) inactivation, which controls rate and extent of ion channel closure during a maintained depolarization. These two processes ensure a rapid, but transient, activation of ion channels in response to membrane potential changes. In addition, the ion conductance activity of voltage-sensitive ion channels is modulated over longer time periods by protein phosphorylation (*2*) and interaction with guanyl nucleotide binding (G) proteins (*3*). This article focuses on three related questions concerning the molecular mechanisms of these voltage-dependent membrane signaling processes. (i) What membrane proteins form the voltage-sensitive ion channels and what are their subunit structures? (ii) What are the common primary structural characteristics of the principal subunits of the ion channel proteins? (iii) How do the structural features of this family of proteins relate to voltage-dependent activation, inactivation, selective ion conductance, and long-term modulation?

Subunit Structures of Voltage-Sensitive Ion Channels

Sodium channels. Biochemical studies of voltage-sensitive Na^+ channels have depended upon use of a number of specific neurotoxins that act at five different receptor sites as molecular probes of channel structure and function as outlined in Table 1 (*4*). Protein components of Na^+ channels were first identified by photoaffinity labeling with photoreactive derivatives of α-scorpion toxins, which act at neurotoxin receptor site 3 (*5*). Polypeptides with masses of approximately 260 kD and 36 kD are covalently labeled in synaptosomal membranes from rat brain. The labeling is considered specific because it was blocked by inhibition of high affinity binding of labeled scorpion toxin by either unlabeled toxin or by depolarization. These two polypeptides, now designated the α and β_1 subunits of the Na^+ channel, are also covalently labeled by photoreactive derivatives of β-scorpion toxins, which act at neurotoxin receptor site 4 (Table 1) (*6*). These results suggest that neurotoxin receptor sites 3 and 4 are located near the contact regions of these two subunits.

Na^+ channels can be solubilized from excitable membranes by treatment with nonionic detergents, detected in solubilized form by high affinity binding of tetrodotoxin or saxitoxin at neurotoxin receptor site 1, and purified by a combination of conventional methods including ion exchange chromatography, lectin-Sepharose

The author is Professor and Chairman of the Department of Pharmacology in the School of Medicine at the University of Washington, Seattle, WA 98195.

Table 1. Neurotoxin receptor sites associated with the Na^+ channel.

Receptor site	Ligands	Physiological effect
1	Tetrodotoxin Saxitoxin μ-Conotoxins	Inhibit ion transport
2	Veratridine Batrachotoxin Aconitine Grayanotoxin	Persistent activation
3	North African α-scorpion toxins Sea-anemone toxins	Inhibit inactivation Enhance persistent activation
4	American β-scorpion toxins	Shift activation
5	*Ptychodiscus brevis* toxin Ciguatoxins	Repetitive firing Persistent activation

chromatography, and sucrose gradient sedimentation (7). Highly purified, functional preparations with well-defined subunit composition have been obtained from mammalian brain and skeletal muscle and electric eel electroplax (*8*). The Na^+ channel from mammalian brain consists of a heterotrimeric complex of α (260-kD), β_1 (36-kD), and β_2 (33-kD) subunits. The β_2 subunit is attached to the α subunit by disulfide bonds. The Na^+ channel from mammalian skeletal muscle contains subunits of 260 kD and 38 kD, analogous to the α and β_1 subunits of the brain Na^+ channel, whereas the Na^+ channel purified from eel electroplax contains only a single 260-kD subunit. These results show that the large α subunits contain the binding site for tetrodotoxin and saxitoxin and suggest that these subunits are the main functional components of Na^+ channels. Reconstitution and expression of functional Na^+ channel activity as described below confirm this conclusion. Although only Na^+ channels from mammalian brain have β_2 subunits, experiments with subunit-specific antibodies indicate that essentially all Na^+ channels in rat brain have these subunits and that the brains of most vertebrates, including the electric eel, contain Na^+ channels with disulfide-linked β_2 subunits (*9, 10*). They are disulfide-linked to α subunits late in the biosynthesis of Na^+ channels, concomitant with insertion into the cell surface. Therefore, it appears that the principal α subunits of Na^+ channels are expressed in association with a variable number of smaller subunits in different excitable tissues.

The overall arrangement of α, β_1, and β_2 subunits in the membrane has been inferred from biochemical experiments. The α and β_1 subunits are covalently labeled by neurotoxins that act from outside the cell (*5, 6*), and all three subunits are heavily glycosylated with up to 30% of their apparent mass due to carbohydrate (*8, 11*), indicating that they are all exposed to the extracellular surface. The α subunit is phosphorylated by adenosine 3′,5′-monophosphate (cAMP)–dependent protein kinase in intact cells or synaptosomes (*12*), indicating that it is exposed at the intracellular surface and therefore is a transmembrane protein. The β_1 and β_2 subunits are also intrinsic membrane proteins that have substantial hydrophobic domains labeled by hydrophobic photoaffinity probes and are preferentially extracted into hydrophobic detergent phases (*13*). These biochemical properties of the three Na^+ channel subunits have led to the proposal of a model of their disposition in the membrane (Fig. 1).

Calcium channels. There are multiple classes of Ca^{2+} channels in most excitable cells (*14*). Ca^{2+} channels that mediate long-lasting Ca^{2+} currents (L-type) are inhibited by three distinct classes of organic Ca^{2+} channel antagonists: dihydropyridines like nifedipine, phenylalkylamines like verapamil, and benzothiazepines like diltiazem (*15*). These structurallly different agents act at three separate, allosterically linked receptor sites (*16*), which are reminiscent of the multiple, interacting neurotoxin receptor sites described for Na^+ channels (Table 1) (*4*). In addition, structurally related dihydropyridine Ca^{2+} channel activators bind at the same dihydropyridine receptor site and favor prolonged activation of the Ca^{2+} channel (*15*). These Ca^{2+} channel modulators bind to the channel with high affinity and have been used as molecular probes to identify and isolate the protein components of dihydropyridine-sensitive (L-type) Ca^{2+} channels in the same way that neurotoxins had been used to identify and isolate Na^+ channels.

Although dihydropyridine-sensitive Ca^{2+} channels are present in essentially all excitable cells, by far the highest concentration of these channels is found in the transverse tubules of skeletal muscle (*17*) where they mediate very slowly activated, long-lasting Ca^{2+} currents (*18*). Experiments on identification and purification of these Ca^{2+} channels have therefore focused on skeletal muscle.

The skeletal muscle Ca^{2+} channel can be solubilized in digitonin or 3-[(cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and purified by a series of conventional purification methods including ion exchange chromatography, lectin-Sepharose chromatography, and sucrose gradient sedimentation (*19, 20*). The purified Ca^{2+} channel is a complex of five polypeptide chains (Fig. 1) (*20, 21*). The central component of the complex is the α_1 subunit with an apparent mass of 175 kD as analyzed by SDS–polyacrylamide gel electrophoresis (*20–26*). This polypeptide contains substantial hydrophobic domains, as assessed by labeling with a hydrophobic photoaffinity probe (*21*), indicating that it has a transmembrane orientation, but it is not heavily glycosylated (*21, 22*). The central α_1 subunit is associated with a disulfide-linked complex of α_2 (143 kD) and δ (27 kD) subunits that are heavily glycosylated (*19–24, 26*), but do not have substantial hydrophobic domains (*21*), β subunits (54 kD) (*20–25*) that are neither glycosylated nor hydrophobic (*21*), and γ subunits (30 kD) (*20–25*) that have both substantial hydrophobic domains and carbohydrate moieties (*21*). The associations among α_1, β, and γ subunits are stronger than with the α_2 subunit, suggesting that the β and γ subunits interact directly with α_1 (Fig. 1). The α_1 and β subunits are substrates for phosphorylation by cAMP-dependent protein kinase (*20–22, 25, 26*), and are the sites of regulation of Ca^{2+} channel ion conductance activity by this enzyme. Although the disulfide-linked complex of α_2 and δ has a combined mass of 170 kD, these polypeptides differ from the α_1 subunits in immunoreactivity (*21, 22, 27*), glycosylation (*21, 22*), and hydrophobicity (*21*), which confirms that they are independent polypeptides. All these components of the Ca^{2+} channel complex are specifically associated as assessed by coimmunoprecipitation with monospecific antibodies and by cosedimentation in sucrose gradients (*20–22, 27, 28*).

The dihydropyridine and phenylalkylamine receptor sites in transverse tubule membranes are located on polypeptides of 145 to 170 kD as assessed by photoaffinity labeling (*29*). Studies with purified Ca^{2+} channels have identified this polypeptide as the α_1 subunit of the Ca^{2+} channel (*20–24*). The α_2 subunit is not labeled by photoreactive derivatives of these organic Ca^{2+} channel antagonists, indicating that it does not contain high affinity receptor sites for them. Because the α_1 subunit contains the Ca^{2+} channel antagonist receptor sites and extensive hydrophobic domains expected of the Ca^{2+} channel protein, it is considered to be the principal functional component of the Ca^{2+} channel, in analogy with the α subunit of the Na^+ channel (Fig. 1).

Although biochemical studies of the dihydropyridine-sensitive Ca^{2+} channel have emphasized experiments on channels from

skeletal muscle, several reports suggest that these channels in brain, heart, and smooth muscle are composed of a similar complex of polypeptides. Antibodies against the skeletal muscle Ca^{2+} channel detect polypeptides analogous to the disulfide-linked α_2-δ complex in brain, heart, and smooth muscle (*21, 30*). Photoaffinity labeling with azidopine and a photoreactive derivative of verapamil has identified a specifically labeled polypeptide of 195 kD in hippocampal and cardiac membranes that may be analogous to the α_1 subunit (*31*). Evidently, Ca^{2+} channels in a range of excitable tissues have a similar subunit structure.

The overall structures of both Na^+ and Ca^{2+} channels (Fig. 1) consist of a single principal subunit expressed in association with a variable number of other polypeptides. The general similarity in biochemical properties of Na^+ channel α subunits and Ca^{2+} channel α_1 subunits has its basis in substantial amino acid sequence similarity between the two proteins. The β_1 subunits of Na^+ channels and the γ subunits of Ca^{2+} channels are similar in size, hydrophobicity, and extent of glycosylation, whereas the other polypeptide components of the two channels do not appear to resemble each other in biochemical properties. Similarities among the primary structures of these other subunits of the voltage-sensitive ion channels may be revealed in future experiments.

Functional Reconstitution

The Na^+- and Ca^{2+}-channel proteins purified in detergent solution on the basis of their high affinity binding of toxins and drugs can be restored to functional form in the purified state by reconstitution into phospholipid vesicles and planar phospholipid bilayers. Purified preparations of these ion channels are first incorporated into phospholipid vesicles by mixing with phospholipids dispersed in detergent and then removing the detergent by dialysis, gel filtration, or adsorption to polystyrene beads. Upon detergent removal, single-walled phospholipid vesicles form that contain the purified proteins incorporated into their bilayer membranes. The functional properties of the incorporated ion channels can then be studied by measuring ion flux into the vesicles with isotopic tracer techniques or fluorescence detection methods after activation by neurotoxins or drugs. These methods allow measurement of the ion conductance activity, ion selectivity, and pharmacological modulation of the whole population of purified ion channels. The properties of single ion channels in the purified population can be analyzed further by fusion of reconstituted vesicles with planar phospholipid bilayers and electrical recording of ionic currents mediated by individual channels. These methods provide a direct measure of the single channel conductance, ion selectivity, and voltage dependence of individual purified ion channels.

Sodium channels. In the initial reconstitution experiments on purified voltage-sensitive ion channels, partially purified Na^+ channel preparations from skeletal muscle, brain, or electroplax were incorporated into phospholipid vesicles. Isotopic flux methods were used to show that these preparations mediate selective ion conductance, which is activated by neurotoxins, such as veratridine and batrachotoxin, acting at neurotoxin receptor site 2 and blocked by tetrodotoxin and saxitoxin (*32*). Subsequently, similar experiments with highly purified preparations of defined subunit composition resulted in recovery of ion conductance activity of a large proportion of the purified Na^+ channels with the ion selectivity and pharmacological properties of native Na^+ channels (*33*). These results demonstrated that the protein components of the isolated channels (α, β_1, and β_2 subunits from brain, α and β subunits from skeletal muscle, or α subunits alone from electroplax) are sufficient to mediate selective, neurotoxin-activated ion flux with the flux rate, ion selectivity, and pharmacological properties expected of native Na^+ channels.

The properties of individual purified Na^+ channels incorporated into macroscopic planar phospholipid bilayers have been studied after activation of the channels by batrachotoxin. Toxin treatment is necessary to prevent inactivation and thereby allow long-term recording of channel openings after the capacitive current transient induced by voltage changes across the large bilayers subsides. In these experiments on Na^+ channels from brain, skeletal muscle, and electroplax, purified Na^+ channels retained the single channel conductance, ion selectivity, and voltage dependence of native Na^+ channels activated by batrachotoxin (*34*). Typical records from the single channel recording experiments of Hartshorne *et al.* (*34*) on purified brain Na^+ channels are shown in Fig. 2. The conductance increase on channel opening corresponds to 25 pS in 500 m*M* Na^+, and the fraction of time that an individual channel is in the open state is strongly voltage-dependent. The probability of opening is 50% at −91 mV and reaches 96% at voltages more positive than −60 mV. The steepness of voltage-dependent activation of the single purified Na^+ channels is consistent with a gating charge movement of 3.8. These results indicate that the voltage-sensing and ion conducting structures of the Na^+ channel are intact in the purified proteins.

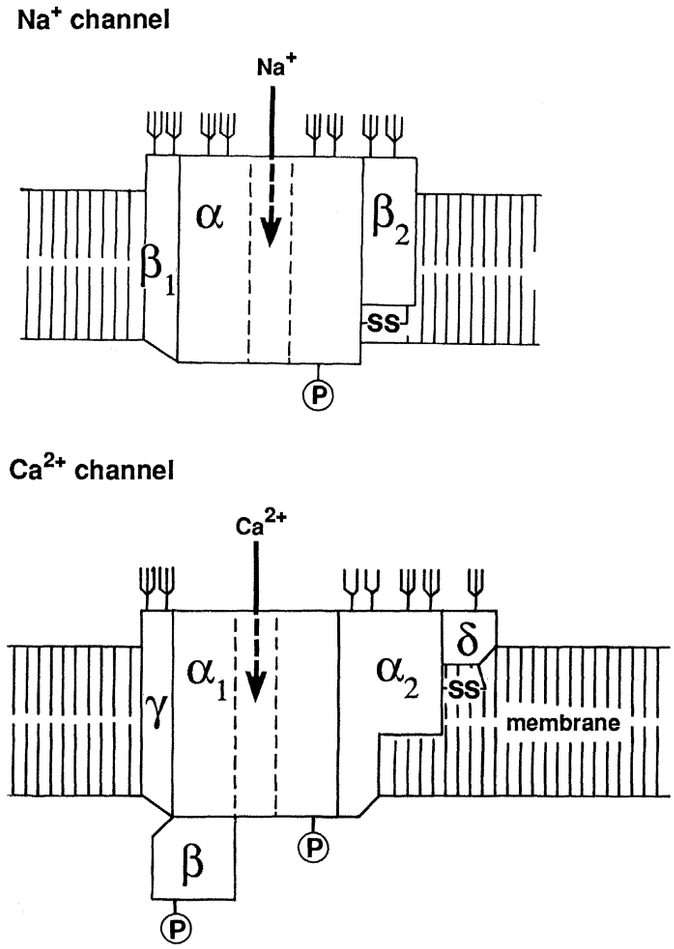

Fig. 1. Subunit structures of Na^+ channels and Ca^{2+} channels. The subunit structure and transmembrane organization of the rat brain Na^+ channel, as defined by Hartshorne *et al.* (*8*), Messner and Catterall (*11*), Costa *et al.* (*12*), and Reber and Catterall (*13*). This drawing is modified from (*47*). The subunit structure of the rabbit skeletal muscle Ca^{2+} channel is illustrated as defined in references (*20*) and (*21*). Disulfide bonds, glycosylation sites, and phosphorylation sites are illustrated. The drawing is modified from (*21*).

The role of the different subunits in the functional properties of the purified Na^+ channels have also been studied by reconstitution methods. The β_1 and β_2 subunits of the rat brain Na^+ channel can be selectively dissociated from the α subunit by treatment with high ionic strength or disulfide reducing agents, respectively (*35*). The β_2 subunit can be removed without effect on the neurotoxin binding, ion flux, single channel conductance, or voltage dependence of the purified channels, whereas removal of β_1 subunits causes loss of all these functional activities. The dissociation of β_1 subunits and loss of functional activities can both be prevented by tetrodotoxin, demonstrating energy coupling between toxin binding and association of β_1 subunits in the functional state of the purified brain Na^+ channel. This functional state can also be stabilized in the absence of β_1 subunits by carbodiimide-induced formation of intramolecular isopeptide bonds with the α subunit itself, suggesting that the role of β_1 subunit in the purified channel is, at least in part, to stabilize the structure of the α subunit (*35*).

The skeletal muscle Na^+ channel is functional in the purified state as a complex of α and β_1-like subunits (*33, 34*) in agreement with the requirement for these subunits, but not the β_2 subunits, for function of the purified brain Na^+ channel. The electroplax is functional in the purified state with only a single α subunit, supporting the view that the α subunit contains most, if not all, of the functional domains of the channel (*33, 34*). Considered together, these results indicate that the α subunit is the principal structural and functional unit of the Na^+ channel and is expressed in different tissues in association with β_1 subunits, which can help to stabilize its normal functional state, and with β_2 subunits, which may have no direct influence on channel function.

Calcium channels. The functional properties of purified dihydropyridine-sensitive Ca^{2+} channels from skeletal muscle have been restored by similar reconstitution methods. Incorporation of highly purified Ca^{2+} channels, prepared in the presence of the Ca^{2+} channel activator BAY K 8644, into phospholipid vesicles results in increased Ca^{2+} conductance of the reconstituted vesicles that is inhibited by Ca^{2+} channel antagonists (*36*). Although the ability to reconstitute Ca^{2+} channel ion conductance correlated closely with the concentration of the α, β, and γ subunits of the purified channel preparation, less than 5% of the purified channels are reconstituted in functional form under these conditions, suggesting that some aspect of the in situ environment may be important for Ca^{2+} channel function. Dihydropyridine-sensitive, or L-type, Ca^{2+} channels in cardiac and neural cells are regulated by intracellular cAMP and cAMP-dependent protein kinase (*2*). In the presence of these agents, the probability of Ca^{2+} channel activation is increased and Ca^{2+} channels are shifted from an inactivated to an activatable state. The Ca^{2+} currents mediated by Ca^{2+} channels in skeletal muscle transverse tubules are also increased by intracellular cAMP and cAMP-dependent protein kinase (*37*). The α_1 and β subunits of purified skeletal muscle of Ca^{2+} channels are good substrates for phosphorylation by cAMP-dependent protein kinase in vitro (*20–26*). Can phosphorylation of the purified Ca^{2+} channel increase its functional activity? Phosphorylation by cAMP-dependent protein kinase increases the initial rate of $^{45}Ca^{2+}$ influx eightfold in reconstituted phospholipid vesicles containing purified Ca^{2+} channels and the number of purified Ca^{2+} channels that are active is increased (*36*). Thus, appropriate phosphorylation appears to be required for functional activity of purified preparations of Ca^{2+} channels.

The functional properties of purified Ca^{2+} channels with well-defined subunit composition have also been examined by incorporation of reconstituted vesicles into planar phospholipid bilayers (*38*). Flockerzi *et al.* observed Ca^{2+} channels with a single channel conductance of 20 pS in 90 m*M* Ba^{2+} that are activated by BAY K 8644 and inhibited by Ca^{2+} channel antagonists, as expected for functional dihydropyridine-sensitive Ca^{2+} channels. The probability of channel opening was increased by phosphorylation by cAMP-dependent protein kinase, providing clear evidence for direct functional modulation of channel gating by cAMP-dependent protein kinase. Hymel *et al.* report more complex gating behavior of these channels with a prominent conductance state of 0.9 pS, which is proposed to represent individual Ca^{2+} channels, and larger conductance states, which are proposed to represent channel oligomers. Both the number of active Ca^{2+} channels and their probability of opening is increased by phosphorylation (*38*). Considered together, the results of ion flux and single-channel recording studies of purified and reconstituted Ca^{2+} channels show that these channels are functional in the purified state and that their functional properties depend critically on cAMP-dependent protein phosphorylation. Purified and reconstituted preparations of Na^+ channels and Ca^{2+} channels will continue to provide valuable tools for analysis of the structure and function of voltage-sensitive ion channels.

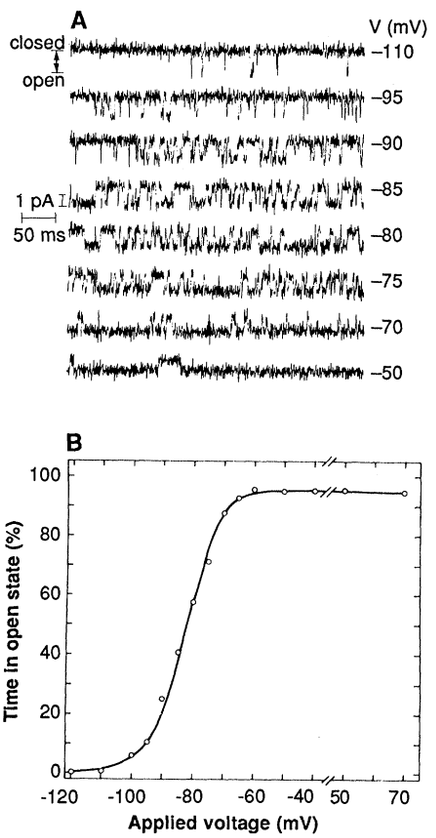

Fig. 2. Single channel activity of purified and reconstituted Na^+ channels. (**A**) The current through a single purified Na^+ channel in a planar phospholipid bilayer (bathed in 0.5*M* NaCl in the cis bath and 0.35*M* NaCl plus 1 μM batrachotoxin in the trans bath) was recorded at the indicated membrane potentials. Channel openings, indicated as downward deflections, correspond to an increase of 25 pS (25×10^{-12} ohms^{-1}) in the conductance of the bilayer. The probability of channel opening increases with depolarization. (**B**) The percent of time that single purified Na^+ channels spend in the open state was determined from records like those in (A) and plotted as a function of membrane potential. The figure is adapted from Hartshorne *et al.* (*34*).

Role of Posttranslational Modifications in Ion Channel Function

Biosynthetic modifications. Both Na^+ channels and Ca^{2+} channels are glycoproteins (*8–11, 19–24*). The Na^+ channel is also modified by fatty acylation and sulfation (*10, 39*). Fifteen to 30% of the mass of Na^+ channel α subunits, approximately 25% of the mass of β_1 subunits, and approximately 25% of the mass of β_2 subunits (*8, 11*) are carbohydrate. Much of the carbohydrate is in complex carbohydrate chains containing large amounts of sialic acid. The electroplax Na^+ channel is more heavily glycosylated than the brain or skeletal muscle Na^+ channels, and a substantial portion of its sialic acid is attached as polysialic acid in α ($2 \rightarrow 8$) linkage (*40*), an unusual form of posttranslational modification in eukaryotes. Because of the

extensive glycosylation of these proteins and the large negative charge contributed by the sialic acid residues, it has been of interest to examine possible roles of these carbohydrate moieties in Na^+ channel biosynthesis and function.

Inhibition of N-linked protein glycosylation with tunicamycin prevents maintenance of a normal number of Na^+ channels in nerve and muscle cells (*9, 41*). In rat brain neurons in cell culture, α subunits are synthesized in nonglycosylated form in the presence of tunicamycin and then rapidly degraded (*9, 10*). The nonglycosylated polypeptide is not further modified by normal fatty acylation and sulfation reactions, is not assembled with β_2 subunits, and probably does not successfully exit the endoplasmic reticulum. In contrast, if N-linked glycosylation is allowed to proceed but subsequent processing and sialylation of the carbohydrate chains is substantially inhibited with castanospermine or swainsonine, Na^+ channels are synthesized, assembled, and inserted into the cell surface normally and have normal high affinity for saxitoxin (*10*). Evidently, core glycosylation of the Na^+ channel subunits is required for correct folding and assembly, but extensive processing and sialylation of the carbohydrate chains are not.

Possible functional roles of the sialic acid residues on Na^+ channels have been examined by removal of sialic acid from purified Na^+ channels from electroplax or brain by treatment with neuraminidase (*42*). Saxitoxin binding to solubilized or reconstituted Na^+ channels and α-scorpion toxin binding to reconstituted Na^+ channels are unaffected. However, single channel currents mediated by purified Na^+ channels in planar bilayers are altered. Unmodified Na^+ channels activated by batrachotoxin have a dominant conductance of 18 ± 2 pS in 100 m*M* Na^+. In contrast, channels with sialic acid removed adopt a range of subconductance states of approximately 5 ± 2, 8 ± 2, and 14 ± 2 pS in addition to the normal state of 18 ± 2 pS. It has been suggested that the negative surface charge contributed by the sialic acid residues on the extracellular side of the Na^+ channel may serve to increase the single channel conductance of the Na^+ channel by increasing the concentration of Na^+ near the entry to the transmembrane pore. The ability of Na^+ channels without sialic acid residues to achieve a normal single channel conductance argues against an important effect of negative surface charge from sialic acid residues on channel conductance. The negative charges of the sialic acid residues may be located a sufficient distance from the entry to the pore so that the local concentration of permanent cations is not markedly increased. In contrast, removal of sialic acid residues appears to destabilize the normal conducting conformation of the pore, allowing it to oscillate among several, probably closely related, ion conductance states with different mean conductance values.

In contrast to the extensive glycosylation of Na^+ channel α subunits, the related α_1 subunit of the skeletal muscle Ca^{2+} channel is not heavily glycosylated (*21, 22*), although there are potential sites of glycosylation in its primary structure. It does not bind lectins specific for either high mannose or complex carbohydrate chains and its apparent mass is not significantly reduced by treatment with neuraminidase or endoglycosidase. The α_2-δ and γ subunits are heavily glycosylated with approximately 30% of their apparent mass composed of carbohydrate (Fig. 1) (*21*). The possible roles of this carbohydrate have not yet been examined. However, the lack of extensive carbohydrate moieties on the α_1 subunit of the Ca^{2+} channel suggests, in agreement with the results of experiments on desialylation of Na^+ channels, that the critical functions of voltage-dependent gating and selective ion transport can be fulfilled without direct participation of carbohydrate groups.

Protein phosphorylation. Na^+ channel α subunits from brain are rapidly phosphorylated in vitro by cAMP-dependent protein kinase and protein kinase C (*12*). In intact synaptosomes and neurons, activation of endogenous cAMP-dependent protein kinase results in phosphorylation of the same sites that can be phosphorylated in vitro (*12*). Without stimulation, these sites contain approximately 2.5 mol of phosphate per mole of Na^+ channel and this concentration is increased to 3.5 mol of phosphate upon stimulation with forskolin or 8-bromo-cAMP. Incubation of synaptosomes with 8-bromo-cAMP under conditions where Na^+ channels become phosphorylated reduces neurotoxin-activated $^{22}Na^+$ influx mediated by Na^+ channels (*12*), consistent with an inhibition of channel activation or enhancement of Na^+ channel inactivation by phosphorylation. Whole-cell voltage clamp recording of Na^+ currents in brain neurons in cell culture in the presence or absence of intracellular cAMP reveals that approximately 20% of Na^+ channels inactivate at more negative membrane potentials in the presence of cAMP (*12*). Na^+ channels from heart, electroplax, and locust nervous system are phosphorylated by cAMP-dependent protein kinase after immunoprecipitation from tissue extracts (*12*). As in the brain, steady-state inactivation of the Na^+ current in the heart, as inferred from measurements of the rate of rise of the action potential or measured directly by whole-cell voltage clamp, is shifted to more negative membrane potentials in the presence of β-adrenergic agonists or other agents that increase intracellular cAMP (*43*). In addition, increases in intracellular cAMP enhance inhibition of action potential conductance by tetrodotoxin in myelinated nerve (*43*). These results suggest an enhancement of Na^+ channel inactivation due to phosphorylation by cAMP-dependent protein kinase. Further electrophysiological studies will be required to clearly document this effect and to show that it is caused by direct phosphorylation of the α subunit of the channel.

Many Ca^{2+} channels and K^+ channels are also regulated by pathways involving protein phosphorylation, suggesting that long-term modulation of ion channel function by phosphorylation is a wide-spread regulatory mechanism (*2*). As described above, purified and reconstituted Ca^{2+} channels are activated by phosphorylation of their α_1 and β subunits by cAMP-dependent protein kinase (*36, 38*), and it is likely that this phosphorylation represents the mechanism of Ca^{2+} channel regulation by β-adrenergic agonists in the heart and other tissues (*2*). However, neither Ca^{2+} channel nor K^+ channels have been shown directly to be phosphorylated in intact cells, and their physiologically significant sites of phosphorylation are not yet known.

Primary Structure

Primary structures of sodium and calcium channels. The availability of purified preparations of voltage-sensitive Na^+ and Ca^{2+} channels that retain normal functional properties provided the basis for isolation of complementary DNA (cDNA) clones encoding the primary structure of the principal subunits of these two ion channels. Noda *et al.* (*44*) screened expression cDNA libraries prepared from eel electroplax messenger RNA (mRNA) both with antibodies against the purified electroplax Na^+ channel α subunit and with oligonucleotides designed to recognize segments of its mRNA sequence. Analysis of the resulting cDNA sequences showed that they encode a large polypeptide of 1832 amino acids containing four repeated domains of 300 to 400 amino acids with an approximately 50% identical or conserved amino acid sequence. Each domain contains multiple hydrophobic segments with the potential for formation of transmembrane α helices. The cDNA clones encoding the corresponding α subunit of the Na^+ channel from rat brain were isolated by screening with cDNA probes encoding the electroplax protein (*45*) or antibodies against the purified protein (*46*). Three different mRNAs encoding Na^+ channel α subunits

were identified in rat brain and fully sequenced to define the rat Na^+ channel subtypes I, II, and III (R_I, R_{II}, and R_{III}) (*45*). Like the electroplax Na^+ channel, these two rat brain Na^+ channel α subunits contain four homologous domains with multiple hydrophobic segments. These proteins are 2008 to 2012 amino acids in length having an insertion of approximately 200 amino acids between the first two homologous domains that is not present in the electroplax channel.

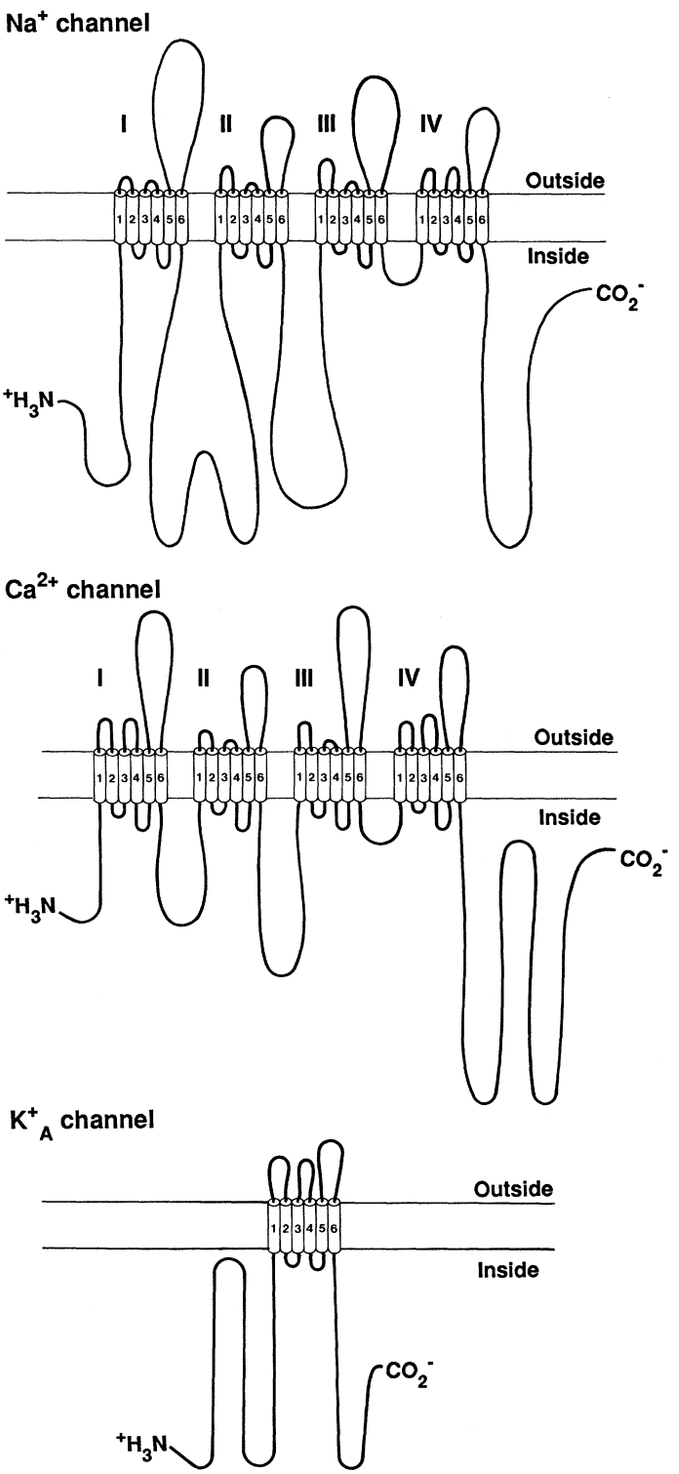

Fig. 3. Proposed transmembrane arrangements of the principal subunits of Na^+, Ca^{2+}, and A-current K^+ channels (K^+_A). The protein folding models for rat brain Na^+ channels (*45*), rabbit skeletal muscle Ca^{2+} channels (*47*), and *Drosophila* A-current K^+ channels (*58*) are presented to illustrate overall sequence similarities. Segment H5, which is proposed to be transmembrane in (*58*), is illustrated as part of an extracellular segment between S5 and S6.

Complementary DNA clones encoding the α_1 and α_2 subunits of the dihydropyridine-sensitive Ca^{2+} channel have also been isolated by screening cDNA libraries with specific antibodies or with oligonucleotides designed to recognize segments of the corresponding mRNA sequence (*47, 47a*). The α_1 subunit consisting of 2005 amino acids has an amino acid sequence that is 29% identical to that of the α subunit of the R_{II} rat brain Na^+ channel and an additional 36% of the residues represent substitutions of chemically similar amino acids. The α_1 subunit contains four homologous domains with multiple hydrophobic segments like the Na^+ channel α subunit. The α_2 subunit (*47a*) consisting of 1106 amino aicds is a unique polypeptide. It is a predominantly hydrophilic protein with 18 potential N-linked glycosylation sites and 2 or 3 potential transmembrane segments. These properties are in close agreement with the previous biochemical studies of this protein (*21, 22, 24*).

Proposed transmembrane organization of the sodium channel α subunit and calcium channel α_1 subunit. Analyses of the hydrophobicity, probable secondary structure, and regional conservation of the amino acid sequence of the α and α_1 subunits of these two ion channels have led to a number of proposed models for their transmembrane folding (*44, 45, 48–51*). Noda *et al.* (*44*) first noted that each of the four homologous domains of the electroplax Na^+ channel contains six regions of probable α-helical structure long enough to be membrane-spanning segments and designated them S1 through S6. They suggested that the segments S1 and S2, which are hydrophobic with occasional hydrophilic residues, and segments S5 and S6, which are uniformly hydrophobic, form the transmembrane structure of the protein, whereas segments S3, which have more numerous charged residues, and segments S4, which are both hydrophobic and positively charged, project from the cytoplasmic surface of the protein (*44*).

After the initial description of the S4 segments as probable α helices with both hydrophobic and positively charged characteristics, specific models of voltage-dependent gating were developed that required that these segments have a transmembrane orientation (*48–50*). Consistent with these proposals, new models of the transmembrane arrangement of the Na^+ channel proteins from eel electroplax and rat brain have been proposed having the S4 segments as one of six or eight transmembrane segments in each homologous domain (*45, 47–50*). The model of the R_{II} Na^+ channel having six membrane-spanning segments per homologous domain (*45*) is illustrated in Fig. 3 with the connecting hydrophilic segments represented in extended form with their length proportional to their amino acid sequence. The membrane-associated segments S7 and S8 in two other structural models (*48, 50*) are proposed to be formed by amino acid residues in the carboxyl terminal half of the proposed extracellular loop between S5 and S6 in each domain in Fig. 3. The inferred amino acid sequence of a homologous *Drosophila* protein, which is presumably a Na^+ channel, also conforms to these proposed structural models (*52*).

The substantial amino acid sequence similarity between the Ca^{2+} channel α_1 subunit and the Na^+ channel α subunits led to a proposal of an analogous transmembrane structure (*47*). The conservation of amino acid sequence in the proposed transmembrane regions and the overall arrangement of hydrophilic and hydrophobic segments is even more striking than the amino acid sequence similarity per se. A model of the transmembrane folding of the Ca^{2+} channel α_1 subunit with six transmembrane segments per homologous domain is illustrated in Fig. 3 (*47*). The most striking difference in overall structure between the principal subunits of the Na^+ channels and the Ca^{2+} channels is the extended hydrophilic domain at the

carboxyl terminus of the Ca^{2+} channel.

Primary structure of an A-current K^+ channel. A novel strategy has been used to clone a *Drosophila* gene encoding a K^+ channel that mediates a rapidly inactivating K^+ current with the kinetic properties of the A current (*1, 53*) in many neurons. The *Shaker* mutation in *Drosophila* results in alteration or loss of a rapidly inactivating K^+ current (*54*). The gene specifying this mutant phenotype was mapped at high resolution to segment 16F of the X chromosome (*55*). The gene was then cloned starting with a DNA probe for a known marker near this chromosomal region and cloning adjacent segments of genomic DNA in a step-by-step manner (*56*), an approach termed "chromosome walking." Successive genomic DNA clones from wild-type and different *Shaker* mutants were characterized by in situ hybridization to identify their chromosomal location and by hybridization to restriction fragments of genomic DNA in DNA blots to identify their location among the restriction fragments from this region of the genome. Genomic fragments originating from the region containing the *Shaker* mutations were then used to isolate cDNA clones from libraries prepared from *Drosophila* head mRNA in order to identify regions of the DNA expressed as mRNA in brain. Clones of interest were identified by hybridization to restriction fragments of *Shaker* genomic DNA with known chromosome breaks. The cDNA clones originated from a 65-kb region of DNA (*56*) containing at least 14 exons, which form at least seven distinct mRNA species by alternative splicing (*57*). This unusually complicated genomic organization appears to give rise to a diversity of K^+ channel proteins from a single genetic locus.

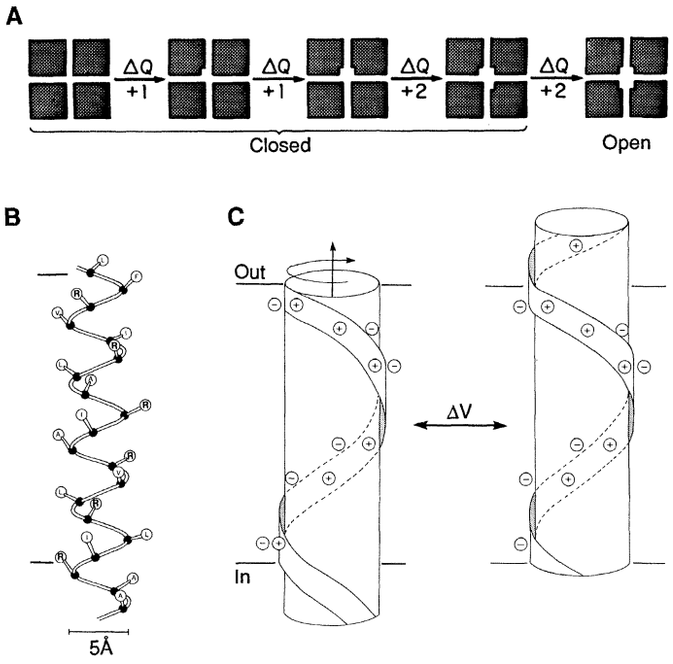

Fig. 4. Sliding helix model of voltage-dependent gating. (**A**) The Na^+ channel α subunit is illustrated from a perspective perpendicular to the plane of the membrane as a square array of four homologous domains surrounding a central transmembrane pore. Depolarization causes a sequential series of voltage-driven conformational changes in the individual domains. After each domain has changed conformation, an open ion channel is formed. Each conformational change is associated with an outward transfer of protein-bound positive gating charge (ΔQ) across the membrane. (**B**) A ball-and-stick, three-dimensional representation of the S4 helix of domain IV of the Na^+ channel. Darkened circles represent the α carbon of each amino acid residue. Open circles show the direction of projection of the side chain of each residue away from the core of the helix. Nonpolar residues are illustrated by their single letter code: F, Phe; A, Ala; I, Ile; L, Leu; V, Val; G, Gly; and T, Thr. Positively charged amino acids are illustrated in bold letters; R, Arg. (**C**) Movement of the S4 helix in response to membrane depolarization. The proposed transmembrane S4 helix is illustrated as a cylinder with a spiral ribbon of positive charge. At the resting membrane potential (left), all positively charged residues are paired with fixed negative charges on other transmembrane segments of the channel and the transmembrane segment is held in that position by the negative internal membrane potential. Depolarization reduces the force holding the positive charges in their inward position. The S4 helix is then proposed to undergo a spiral motion through a rotation of approximately 60° and an outward displacement of approximately 5 Å. This movement leaves an unpaired negative charge on the inward surface of the membrane and reveals an unpaired positive charge on the outward surface to give a net charge transfer (ΔQ) of +1. Note that all intramembranous positive charges are paired in both conformations. In domains III and IV, there are enough arginine residues to give a gating charge movement of +2.

The protein encoded by one mRNA from this gene consists of 616 amino acids. One segment is 27% identical (plus 20% conservative substitutions) to a 121–amino acid segment of domain IV of the electroplax Na^+ channel (*58*). Analysis of the sequence for hydrophobicity reveals six or seven possible membrane-spanning segments. The fourth of these segments has striking homology with the S4 segments of the Na^+ channel and the overall arrangement of the proposed transmembrane segments is similar to that within each of the four homologous domains of the Na^+ channel. To emphasize the similarity to Na^+ and Ca^{2+} channels, the transmembrane arrangement of this polypeptide is drawn with six membrane-spanning segments in Fig. 3, rather than with seven as in (*56*). This representation illustrates the similarity between this protein and the individual homologous domains of the Na^+ and Ca^{2+} channels. Evidently, this protein component of the *Drosophila* A-current K^+ channel corresponds in structure to a single domain of the principal subunits of the Na^+ or Ca^{2+} channels (Fig. 3).

Evolutionary relations among voltage-sensitive ion channels. Voltage-sensitive K^+ channels are common among yeast and other protists, voltage-sensitive Ca^{2+} channels are found in higher protozoa, and Na^+ channels are found only in multicellular organisms (*1*). Thus, it has been proposed that K^+ channels were the original voltage-sensitive ion channels and that Ca^{2+} and Na^+ channels arose from them during evolution (*1*). This concept is supported by the primary structures and proposed secondary structures of the voltage-sensitive ion channels for which amino acid sequences have been determined. There is striking homology among the polypeptides. Moreover, the four homologous domains of the Na^+ and Ca^{2+} channel subunits suggest that they may have arisen from an ancestral protein representing only one homologous domain. The A-current K^+ channel has the structure expected for such an ancestral protein. The complex alternative splicing of its gene provides a basis for generation of substantial diversity within a single class of voltage-sensitive ion channels.

Expression of Functional Ion Channels from mRNA

Sodium channels and calcium channels. Injection of mRNA from excitable tissues into *Xenopus* oocytes results in expression of voltage-sensitive Na^+, Ca^{2+}, and K^+ channels (*59*). Injection of a high molecular weight fraction of mRNA from rat brain is sufficient for functional expression of Na^+ channels, which indicates that only mRNA encoding α subunits is required (*60, 61*). Successful expression of Na^+ channels from α subunit mRNA isolated by hybrid selection (*61*) and of R_{II} mRNA made from cloned cDNA (*46, 62*) also supports this conclusion. Assuming that *Xenopus* oocytes do not produce β_1 and β_2 subunits themselves, these results show conclusively that the α subunits of R_{II} Na^+ channels are functionally autonomous when separately expressed in oocytes. In contrast, these α subunits are expressed in association with β_1 and β_2 subunits in neurons (*9*), and a complex of α and β_1 subunits of rat brain Na^+

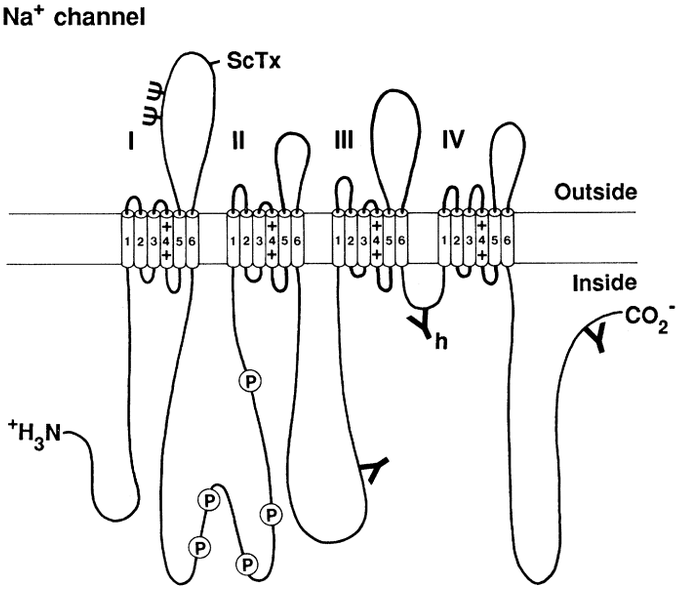

Fig. 5. A functional map of the Na^+ channel α subunit. The transmembrane folding model of the α subunit is depicted with experimentally demonstrated sites of cAMP-dependent phosphorylation (P), interaction of site-directed antibodies that define transmembrane orientation (➢), covalent attachment of α-scorpion toxins (ScTx), glycosylation (ψψ), and modulation of channel inactivation (h).

channels is the minimal unit capable of retaining Na^+ channel function during purification and reconstitution (*35*). The requirement for association of β_1 subunits with α subunits to maintain a functional form of the α subunit in the purified state is likely to reflect a structural interaction between the two subunits that stabilizes the functional form of the purified α subunit.

The successful expression of the R_{II} and R_{III} Na^+ channel subtypes from mRNA encoding only the α subunit (*61, 62*) suggests that the corresponding protein components of the R_I and electroplax Na^+ channels and the dihydropyridine-sensitive Ca^{2+} channel are the principal functional subunits of those proteins as well. However, the R_I mRNA is expressed poorly in *Xenopus* oocytes (*62*) and functional expression of cloned cDNAs encoding the electroplax Na^+ channel and the dihydropyridine-sensitive Ca^{2+} channel in *Xenopus* oocytes has not been reported. Therefore, autonomous expression and function may not be a general characteristic of these homologous polypeptides.

Although the α_1 subunit of the Ca^{2+} channel is not autonomously expressed in *Xenopus* oocytes, successful functional expression of its cloned DNA has been achieved in mutant *mdg* mice, which lack functional dihydropyridine-sensitive Ca^{2+} channels (*63*). Microinjection of cDNA in an expression vector driven by the simian virus 40 (SV40) promoter into the nuclei of myotubes developing in cell culture restores both dihydropyridine-sensitive Ca^{2+} channels and excitation-contraction coupling in these cells. Thus, the α_1 subunits are essential for synthesis of dihydropyridine-sensitive Ca^{2+} channels. However, since the *mdg* muscle cells presumably contain normal complements of the other Ca^{2+} channel subunits, cDNA encoding α_1 subunits may not be sufficient to express Ca^{2+} channel function by itself.

Although high molecular weight mRNA from rat brain and mRNA encoding only the α subunit of R_{II} Na^+ channels can direct synthesis of functional Na^+ channels in *Xenopus* oocytes, the functional properties of these channels are not completely normal. For Na^+ channels expressed from high molecular weight brain mRNA or from mRNA transcribed from cloned DNA, the time course of inactivation is much slower in comparison with Na^+ channels expressed in oocytes from total rat brain mRNA (*46, 61*) or R_{II} Na^+ channels in brain neurons (*12*). Coinjection of a low molecular weight mRNA fraction from rat brain restores the time course of channel inactivation to normal and increases the cell surface expression of functional Na^+ channels threefold (*46*). Evidently, proteins encoded by low molecular weight mRNA species can modulate the functional properties and cell surface expression of R_{II} α subunits. These mRNAs might encode β_1 or β_2 subunits or other enzymes or factors that alter α subunit function by posttranslational modification.

Potassium channels. Because the A-current K^+ channel gene encodes a protein homologous to only a single domain of the Na^+ and Ca^{2+} channel structure, examination of its capacity for expression of functional K^+ channels is of great interest. Injection of mRNA encoding this single protein is sufficient for expression of the A current in *Xenopus* oocytes (*64*). Two different mRNAs made by alternative splicing are able to direct synthesis of K^+ channels with subtle differences in time course and extent of inactivation of the A current. These results are consistent with the idea that this single polypeptide can form a tetramer to carry out the voltage-dependent gating and ion conductance activities characteristic of the Na^+ and Ca^{2+} channel principal subunits, which are four times as large. These results provide further support for the proposal that a K^+ channel gene encoding a similar single domain was the ancestral voltage-sensitive ion channel.

Structural Models of Ion Channel Function

Determination of the primary structures of the principal subunits of the voltage-sensitive ion channels has provided a molecular template for design of models and experiments to probe the relation between ion channel structure and function at higher resolution than previously possible. Because the primary structure of Na^+ channel α subunits was determined first, most of the work to date has focused on them.

The transmembrane pore. Na^+ channel α subunits are sufficient to form an active, voltage-gated ion channel (*46, 61, 62*). Nicotinic acetylcholine receptors and gap junction proteins from high conductance transmembrane pores in the center of a symmetric array of five homologous or six identical transmembrane subunits, respectively (*65*). Therefore, the presence of four homologous domains in the primary structure of the Na^+ channel α subunit led to the proposal that the transmembrane pore of the Na^+ channel is formed in the center of a pseudosymmetric square array of these four homologous transmembrane domains (Fig. 4A) (*44*). All subsequent models of Na^+ channel structure have incorporated this basic architecture (*48–51*). The strong conservation of the structure of the corresponding homologous domains in the amino acid sequences of the α_1 subunit of Ca^{2+} channels and the A-current K^+ channel support the concept that they form transmembrane pores with a similar structure. Formation of a transmembrane pore in the center of a pseudosymmetric array of homologous transmembrane units may be a general structural motif among high conductance ion channels.

The hydrophobic segments within the homologous domains of all three voltage-sensitive ion channels are predicted to form α-helical secondary structures (*44, 47–51*). The walls of the transmembrane pore are therefore thought to be formed by α-helical segments from each homologous domain, as previously proposed for nicotinic acetylcholine receptors (*65*). A pore formed from four such transmembrane α helices would have an internal diameter consistent with the limiting size of 3 by 5 Å inferred for the Na^+ channel from measurements of the permeabilities of organic cations of different

Table 2. Conservation of S4 of voltage-sensitive ion channels. Amino acid sequences of S4 segments from rat brain (R_I or R_{II}) Na^+ channels (*45*), rabbit skeletal muscle Ca^{2+} channels (*47*) and *Drosophila* A-current (K^+_A) K^+ channels (*58*) are compared. Positively charged amino acids are in bold type (R, Arg; K, Lys). Identical amino acids are indicated by solid lines; conservative substitutions [(M, Met; V, Val; I, Ile; L, Leu); (A, Ala; G, Gly); (S, Ser; T, Thr; Q, Gln; N, Asn); (K, Lys; R, Arg); and (D, Asp; E, Glu)] are indicated by dashed lines.

Segment	Channel	Amino acid sequence
IS4	Na^+	S A L **R** T F **R** V L **R** A L **K** T I S V I P G L **K**
	Ca^{2+}	**K** A L **R** T F **R** V L **R** P L **R** V L S G V P S L Q
IIS4	Na^+	G L S V L **R** S F **R** L L **R** V F **K** L A **K** S W P
	Ca^{2+}	L G I S V L **R** C I **R** L L **R** L F **K** I T **K** Y W T
IIIS4	Na^+	G A I **K** S L **R** T L **R** A L **R** P L **R** A L S **R** F E
	Ca^{2+}	S V V **K** I L **R** V L **R** A L **R** P L **R** A I N **R** A **K**
IVS4	K^+_A	**R** V I **R** L V **R** V F **R** I F **K** L S **R** H S **K** G L Q
	Na^+	**R** V I **R** L A **R** I G **R** I L **R** L I **K** G A **K** G I **R**
	Ca^{2+}	I S S A F F **R** L F **R** V M **R** L I **K** L L S **R** A E

sizes (*1*). Which transmembrane α helices are likely to form the walls of the transmembrane pore of the Na^+ channel? Because they lack hydrophilic groups to accommodate the passage of cations, the S5 and S6 helical segments have not been proposed as pore-forming segments. Different models have incorporated the S2 (*44, 45, 46*), S3 (*50*), a combination of S4 and S7 (*48*), or a combination of the hydrophilic segment between domains II and III and the S4 helical segments (*51*) as pore-forming transmembrane segments. One approach to this question is to examine the ability of proposed pore-forming transmembrane segments to conduct ions across planar phospholipid bilayers (*66*). A synthetic peptide corresponding to the S3 segment of domain I of the R_I Na^+ channel forms cation-selective channels with a predominant conductance of 20 pS and minor conductances of 10 and 50 pS. Presumably the cation-selective channels formed by this peptide result from association of multiple molecules oriented perpendicular to the plane of the membrane in α-helical conformation. Although these results are in agreement with the idea that this sequence of amino acids could form the walls of a cation-selective pore, a wide range of peptides can form ion channels in planar bilayers (*67*), making it difficult to reach definite conclusions from experiments of this kind.

Voltage-dependent activation. The steep voltage dependence of activation of the voltage-sensitive ion channels is their most unique characteristic. Steep voltage dependence requires that the channels have charged amino acid residues or strongly oriented dipoles within the electric field of the phospholipid bilayer. For example, the steepness of voltage-dependent activation of Na^+ channels requires the movement of four to six protein-bound positive charges from the inner surface of the bilayer membrane to the outer surface during activation, or movement of a larger number of charges a proportionally smaller distance across the membrane (*1, 68*). 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 Na^+ channels 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 (*68*). This movement of gating charge begins immediately upon depolarization of the membrane, is largely complete before movement of ionic current through the open channel is detected, and is blocked if the Na^+ channel is first inactivated before depolarization.

The requirement for transmembrane movement of multiple charges during Na^+ 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, were first noted in the amino acid sequence of the electroplax Na^+ channel (*44*). Conservation of this amino acid sequence among different voltage-sensitive ion channels, first noted for Na^+ channels from electroplax and brain (*45, 49*), is striking across a broad range of ion channels from diverse species (Table 2).

Several authors have independently proposed that these S4 segments have a transmembrane orientation and are the gating charges or voltage sensors of the Na^+ channel (*45, 48–50*). The "sliding helix" or "helical screw" model of voltage-dependent gating is illustrated in Fig. 4B (*48, 49*). 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. These residues 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. 4). 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. Upon 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 (Fig. 4). This sliding motion of the S4 helix transfers the equivalent of a full positive charge across 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 Na^+ channel resulting in the transfer of at least four gating charges across the membrane and, finally, in activation of the ion channel (Fig. 4). The requirement for sequential conformational changes in the four domains before activation of the ion channel fits with the expected kinetics of Na^+ channel activation in which gating charge moves immediately upon depolarization, whereas ionic current begins to flow only after a significant lag time (*68*). The presence of four domains in the Na^+ channel structure, each of which contains an S4 segment, immediately accounts for a gating charge movement of +4. The S4 segments of domains III and IV of Na^+ channels contain enough positively charged residues to allow outward movement of two gating charges, resulting in a total gating charge movement of +6 by this mechanism.

The sliding helix model proposes that the S4 helix, a unique, highly conserved structural feature of the voltage-sensitive ion channels, initiates voltage-dependent gating. Although this model has many appealing features, its only experimental support comes from the strong conservation of these structural elements among the voltage-sensitive ion channels. Work in progress in several laboratories aims to develop experimental tests of this model of voltage-dependent gating through site-directed mutation and expression of ion channels with altered function and through development of site-directed antibodies, which may interrupt critical aspects of voltage-dependent functions.

Inactivation. The ion conductance activity of voltage-sensitive ion channels is transient because prolonged activation causes transition to an inactive state that cannot be activated by further depolarization. The mechanisms of inactivation of the voltage-sensitive channels differ significantly. Inactivation of Na^+ channels is an intrinsically voltage-independent or weakly voltage-dependent process that derives its strong voltage-dependence by coupling to the voltage-dependent conformational changes leading to channel activation

(*68–70*). Inactivation can be slowed by neurotoxins acting at the extracellular surface of the channel (*4*) and can be completely eliminated by proteolytic enzymes and group-specific reagents acting at the intracellular surface of the channel (*70, 71*); therefore inactivation must involve a transmembrane conformational change in the Na^+ channel protein. The lack of intrinsic voltage dependence and the complete removal of inactivation by internally applied proteolytic enzymes have led to the development of a model of inactivation in which a protein component on the intracellular surface of the channel occludes the intracellular opening of the activated transmembrane pore leading to inactivation (*68*). We have therefore studied the physiological effects of antibodies directed against segments of the proposed intracellular domains of the Na^+ channel α subunit.

Because Na^+ channels in different tissues have similar inactivation properties, one would expect strong conservation of the amino acid segments that play a primary role in inactivation. Inspection of the amino acid sequences of the proposed major intracellular segments of the electroplax, brain, and *Drosophila* Na^+ channels reveals striking conservation of the relatively short intracellular segment connecting domains III and IV. A model of inactivation in which this conserved segment forms a helical hairpin that occludes the intracellular opening of the transmembrane pore has been proposed (*48*). Because segments of the channel protein involved in channel inactivation are accessible to macromolecular reagents, their functional activities might be inhibited by appropriate site-directed antibodies. Antibodies against a synthetic peptide corresponding to this segment of the α subunit (Fig. 5) recognize Na^+ channels in brain, skeletal muscle, heart, and locust nerve cord, extending the broad range of Na^+ channels in which this amino acid sequence is highly conserved (*72*). These antibodies to peptides slow Na^+ channel inactivation without markedly altering the voltage dependence of Na^+ channel activation or inactivation when applied to the intracellular surface of Na^+ channels in skeletal muscle cells (*72*). Antibody effects appear more rapidly at a membrane potential of −110 mV than at −70 mV, suggesting that the antibody binds preferentially to the "not inactivated" states of Na^+ channels. The extent of slowing of inactivation is greatest during strong depolarizations to positive membrane potentials, indicating that pathways of inactivation that are most important at positive potentials are preferentially blocked. These results support a direct role for this highly conserved intracellular segment in Na^+ channel inactivation and suggest that conformational changes associated with Na^+ channel inactivation alter the accessibility of this amino acid sequence to antibodies.

Antibodies against a peptide corresponding to the S4 segment of domain I of the Na^+ channel also modify inactivation (*73*). The time course of inactivation is accelerated and the voltage dependence is shifted to more positive membrane potentials. Because antibodies directed against the S4 segment of domain I (*73*) shift the voltage dependence of inactivation when added outside the cell, and antibodies directed against the intracellular region between domains III and IV also show inactivation and bind in a voltage-dependent manner (*72*), conformational changes specifying Na^+ channel inactivation must be propagated through the protein structure between these two sites on opposite sides of the membrane.

Phosphorylation sites. Cyclic AMP–dependent phosphorylation modifies the functional properties of the Na^+ channel by shifting the voltage dependence of steady state Na^+ channel inactivation without altering the time course of inactivation (*12, 43*). The location of the phosphorylation sites responsible for this physiological effect has been determined with sequence-directed antibodies, which recognize the corresponding amino acid sequences of the α subunit (*74*). The five major cAMP-dependent phosphorylation sites are on a single large intracellular segment of the α subunit between homologous domains I and II (Fig. 5). The segment containing these phosphorylation sites is not present in the electroplax Na^+ channel, suggesting that the functional effects of phosphorylation of these sites may not be observed for all Na^+ channels.

Receptor sites. Because the neurotoxins and drugs that alter voltage-sensitive ion channel function have very specific effects, location of their sites of action will be a major contribution to understanding the functional architecture of these proteins. Alpha-scorpion toxins bind to neurotoxin receptor site 3 on the extracellular surface of the Na^+ channel and slow Na^+ channel inactivation (*4*). Photoaffinity labeling studies have shown that this site is located on the α subunit (*5*). The site of covalent attachment of α-scorpion toxin to the α subunit of the rat brain Na^+ channel has been investigated with site-directed antibodies (*75*). Na^+ channel α subunits, having covalently bound α-scorpion toxin, were cleaved with a series of proteases, and the resulting labeled fragments were analyzed by immunoprecipitation with a panel of antibodies against 20 amino acid segments of the protein. A heavily glycosylated fragment of 50 kD contained the bound scorpion toxin and was identified by immunoprecipitation as the region of homologous domain I from the intracellular limit of segment S1 to the extracellular limit of segment S6. Further cleavage with cyanogen bromide led to isolation of a 14-kD fragment containing covalently bound α-scorpion toxin. Immunoprecipitation with site-directed antibodies showed that this fragment is derived from the extracellular segment between transmembrane segments S5 and S6 of domain I (Fig. 5). Neurotoxin receptor site 3, at which α-scorpion toxins and sea-anemone toxins specifically affected Na^+ channel inactivation, is therefore located near the largest extracellular segment of domain I in the three-dimensional structure of the Na^+ channel. Recent results have therefore defined three sites from which the inactivation of Na^+ channels can be modified. The time course of inactivation can be slowed by scorpion toxins acting at a site in the extracellular portion of domain I and by antibodies acting at a site in the intracellular segment between domains III and IV (Fig. 5). The voltage dependence of steady-state inactivation can be shifted by phosphorylation of sites in the intracellular segment between domains I and II. Evidently, multiple regions of the channel structure interact in mediating the process of voltage-dependent inactivation of Na^+ channels.

Transmembrane Topology of the Voltage-Sensitive Ion Channels

The models for transmembrane folding of the principal subunits of the Na^+, Ca^{2+}, and K^+ channels (Fig. 2) were derived from computer-assisted analysis of hydrophobicity and secondary structure together with the assumptions that all four homologous domains should adopt similar transmembrane arrangements and that the amino terminal should be intracellular because no signal sequence was detected. Several pieces of experimental evidence now support the indicated arrangement of the major hydrophilic segments of the Na^+ channel (Fig. 5). Electron microscopic localization of bound antibodies directed against synthetic peptides corresponding to 20 amino acid sequences of the carboxyl-terminal hydrophilic segment and the hydrophilic segment between domains II and III of the electroplax Na^+ channel shows that they are both located on the intracellular surface of the membrane (*76*). Cyclic AMP–dependent phosphorylation of five sites in the hydrophilic segment between homologous domains I and II of the rat brain Na^+ channel in intact neurons defines this segment as intracellular (*74*). Modulation of Na^+ channel inactivation by intracellular perfusion of antibodies against the hydrophilic segment between homologous

domains III and IV places this segment on the intracellular surface of the membrane as well (*72*). These results provide four points of reference on the intracellular surface of the channel that agree with the overall orientation in Fig. 5. Because the four homologous domains are considered by nearly all investigators to adopt similar transmembrane orientations (*44, 45, 47–50*), the amino-terminal hydrophilic segment is also likely to be intracellular.

Each homologous domain of the Na^+ channel has a major hydrophilic segment that is proposed to be extracellular between the proposed transmembrane segments S5 and S6 (Fig. 5). This segment of domain I is covalently labeled by α-scorpion toxin (*75*). Limited cleavage of the Na^+ channel with proteases results in generation of a 50-kD fragment containing most of domain I and covalently attached α-scorpion toxin (*75*). Treatment with neuraminidase reduces the apparent size of this fragment from 70 to 50 kD, indicating multiple sites of glycosylation. Seven possible sites of N-linked glycosylation (amino acid sequence Asn X Ser/Thr) are clustered between S5 and S6. Because this segment is glycosylated and is labeled by α-scorpion toxins, which act from outside the cell, it must be extracellular. Although direct evidence is not available at present, the strong homology among the four domains indicates that segments between S5 and S6 are likely to be at least partially extracellular in all four domains, as proposed in nearly all of the transmembrane folding models (*45, 47–51*) (Fig. 5). The results outlined here all support the location of the major hydrophilic domains as proposed in the general transmembrane folding schemes developed on the basis of computer analysis of amino acid sequence. However, clear definition of the topology of the voltage-sensitive ion channels requires determination of the transmembrane orientation of each of the segments within the homologous domains. Direct experimental evidence on this problem is still lacking.

Conclusion

Experimental work of the past several years has succeeded in identification and purification of the protein components of the voltage-sensitive ion channels and restoration of their functional activity in the purified state. Subunit structures of the Na^+ and Ca^{2+} channels include a single principal subunit containing the major functional domains of the channels in association with a variable number of other polypeptides with incompletely understood functional roles. The primary structures of the principal subunits of one example of the three major classes of voltage-sensitive ion channels—the Na^+ channels, Ca^{2+} channels, and K^+ channels—have been determined by cDNA cloning and sequencing techniques, and these experiments have shown that these proteins are members of a gene family that probably evolved from a common ancestral protein. Common structural features of these principal subunits have provided the basis for development of several specific and testable hypotheses for the structural basis of their functional properties. Substantial progress has been made toward development of a functional map of the α subunit of the Na^+ channel, and these experimental results provide support for specific aspects of these functional hypotheses. However, the size and complex structural and functional features of these proteins suggest that much more experimental work is needed before an understanding of the molecular basis of electrical excitability is achieved. Because of the importance of electrical signaling in information transmission and processing in the nervous system, in the actions of many classes of pharmacological agents, and in regulation of function of a broad range of cell types, development of a detailed understanding of the mechanisms of electrical excitability will continue to be a major goal in cellular and molecular biology.

REFERENCES AND NOTES

1. B. Hille, *Ionic Channels of Excitable Membranes* (Sinauer, Sunderland, MA, 1984).
2. H. Reuter, *Nature* **301**, 569 (1983); I. B. Levitan, *J. Memb. Biol.* **87**, 177 (1985); L. K. Kaczmarek, *Trends Neurosci.* **10**, 30 (1987); R. Tsien *et al.*, *J. Mol. Cell. Cardiol.* **18**, 691 (1986); S. Rossie and W. A. Catterall in *The Enzymes* (Academic Press, New York, 1987), vol. 18, pp. 335–358; W. Trautwein, M. Kameyama, J. Hescheler, F. Hofmann, *Prog. Zool.* **33**, 163 (1986).
3. P. Pfaffinger, J. Martin, D. D. Hunter, N. M. Nathanson, B. Hille, *Nature* **317**, 536 (1985); G. Breitwieser and G. Szabo, *ibid.*, p. 538; W. Rosenthal and G. Schultz, *Trends Pharmacol. Sci.* **8**, 351 (1987); A. M. Brown and L. Birnbaumer, *Am. J. Physiol.* **254**, H401 (1988).
4. W. A. Catterall, *Annu. Rev. Pharmacol. Toxicol.* **20**, 15 (1980); *ISI Atlas of Science: Pharmacology* **2**, 190 (1988); T. Narahashi, *Physiol. Rev.* **54**, 813 (1984); J. Ritchie and R. Rogart, *Rev. Physiol. Biochem. Pharmacol.* **79**, 1 (1977); E. Albuquerque and J. Daly, *The Specificity and Action of Animal, Bacterial and Plant Toxins: Receptors and Recognition Series B*, P. Cuatrecasas, Ed. (Chapman and Hall, London, 1976) pp. 299–338.
5. D. Beneski and W. A. Catterall, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 639 (1980); R. Sharkey, D. Beneski, W. A. Catterall, *Biochemistry* **23**, 6078 (1984); E. Jover, A. Massacrier, P. Cau, M.-F. Martin, F. Couraud, *J. Biol. Chem.* **263**, 1542 (1988).
6. H. Darbon *et al.*, *Biochem. Biophys. Res. Commun.* **115**, 415 (1983); J. Barhanin, A. Schmid, A. Lombet, L. Wheeler, M. Lazdunski, *J. Biol. Chem.* **258**, 700 (1983); F. Couraud, E. Jover, J. Dubois, H. Rochat, *Toxicon* **20**, 9 (1982).
7. W. Agnew, S. Levinson, J. Brabson, M. Raftery, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 2606 (1978); R. Barchi, S. Cohen, L. Murphy, *ibid.* **77**, 1306 (1980); W. A. Catterall, C. Morrow, R. Hartshorne, *J. Biol. Chem.* **254**, 11379 (1979).
8. W. Agnew, A. Moore, S. Levinson, M. Raftery, *Biochem. Biophys. Res. Commun.* **92**, 860 (1980); J. Miller, W. Agnew, S. R. Levinson, *Biochemistry* **22**, 462 (1983); *Proc. Natl. Acad. Sci. U.S.A.* **78**, 4620 (1981); R. Hartshorne and W. A. Catterall, *J. Biol. Chem.* **259**, 1667 (1984); R. Hartshorne, D. Messner, J. Coppersmith, W. A. Catterall, *ibid.* **257**, 13888 (1982); R. Barchi, *J. Neurochem.* **36**, 2097 (1983); J. M. Casadei, R. D. Gordon, R. L. Barchi, *J. Biol. Chem.* **261**, 4318 (1986).
9. J. W. Schmidt, S. Rossie, W. A. Catterall, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 4847 (1985); J. W. Schmidt and W. A. Catterall, *Cell* **46**, 437 (1987); D. A. Wollner, D. J. Messner, W. A. Catterall, *J. Biol. Chem.* **262**, 14709 (1987).
10. J. W. Schmidt and W. A. Catterall, *J. Biol. Chem.* **262**, 13713 (1987).
11. D. J. Messner and W. A. Catterall, *ibid.* **260**, 10597 (1985); E. V. Grishin, V. A. Kovalenko, V. N. Pashkov, O. G. Shamotienko, *Biol. Membr.* **1**, 858 (1984); L. W. Elmer, B. J. O'Brien, T. J. Nutter, K. J. Angelides, *Biochemistry* **24**, 8128 (1985); R. H. Roberts and R. L. Barchi, *J. Biol. Chem.* **262**, 2298 (1987).
12. M. Costa, J. Casnellie, W. A. Catterall, *J. Biol. Chem.* **257**, 7918 (1982); M. Costa and W. A. Catterall, *ibid.* **259**, 8210 (1984); *Cell Mol. Neurobiol.* **4**, 291 (1984); S. Rossie and W. A. Catterall, *J. Biol. Chem.* **262**, 12735 (1987); J. Coombs, T. Scheuer, S. Rossie, W. A. Catterall, *Biophys. J.* **53**, 542a (1988); D. Gordon, D. Merrick, D. Wollner, W. A. Catterall, *Biochemistry*, **27**, 7032 (1988).
13. B. F. X. Reber and W. A. Catterall, *J. Biol. Chem.* **262**, 11369 (1987).
14. C. M. Armstrong and D. R. Matteson, *Science* **227**, 65 (1985); E. Carbone and H. D. Lux, *Nature* **310**, 501 (1984); M. Nowycky, A. Fox, R. W. Tsien, *ibid.* **316**, 440 (1985); B. P. Bean, *J. Gen. Physiol.* **86**, 1 (1985); G. Cota and E. Stefani, *J. Physiol. (London)* **370**, 151 (1986); L. Byerly and S. Hagiwara, in *Calcium and Ion Channel Modulation*, A. Grinnell, D. Armstrong, M. Jackson, Eds. (Plenum, New York, in press).
15. D. J. Triggle and R. A. Janis, *Annu. Rev. Pharmacol. Toxicol.* **27**, 347 (1987); M. Schramm, G. Thomas, R. Towart, G. Franckowiak, *Nature* **303**, 535 (1983); P. Hess, J. Lansman, R. W. Tsien, *ibid.* **311**, 538 (1984); S. Kokubun and H. Reuter, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 4824 (1984).
16. H. Glossmann, D. R. Ferry, J. Striessnig, A. Goll, K. Moosburger, *Trends Pharmacol. Sci.* **8**, 95 (1987); H. Glossmann, R. Ferry, A. Goll, J. Striessnig, M. Schober, *J. Cardiovasc. Pharmacol.* **7**, 520 (1985); M. Garcia, V. King, P. Siegl, J. Reuben, G. Kaczorowski, *J. Biol. Chem.* **261**, 8146 (1986).
17. H. Glossmann, D. Ferry, C. Boschek, *Naunyn-Schmiedeberg's Arch. Pharmacol.* **323**, 1 (1983); M. Fosset, E. Jaimovich, E. Delpont, M. Lazdunski, *J. Biol. Chem.* **258**, 6086 (1983).
18. J. Sanchez and E. Stefani, *J. Physiol. (London)* **283**, 197 (1978); W. Almers, E. McCleskey, P. Palade, in *Calcium in Biological Systems*, M. Rubin, G. Weiss, J. Putney, Eds. (Plenum, New York, 1985), pp. 321–330.
19. M. Borsotto, J. Barhanin, M. Fosset, M. Lazdunski, *J. Biol. Chem.* **260**, 14255 (1985); M. Borsotto, J. Barhanin, R. Norman, M. Lazdunski, *Biochem. Biophys. Res. Commun.* **122**, 1357 (1984).
20. B. M. Curtis and W. A. Catterall, *J. Biol. Chem.* **258**, 7280 (1983); *Biochemistry* **23**, 2113 (1984); *Proc. Natl. Acad. Sci. U.S.A.* **82**, 2528 (1985).
21. M. Takahashi, M. J. Seagar, J. Jones, B. F. X. Reber, W. Catterall, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 5478 (1987); M. Takahashi and W. A. Catterall, *Biochemistry* **26**, 5518 (1987); W. A. Catterall, M. J. Seagar, M. Takahashi, *J. Biol. Chem.* **263**, 3535 (1988).
22. A. T. Leung, T. Imagawa, K. P. Campbell, *J. Biol. Chem.* **262**, 7943 (1987); A. H. Sharp, T. Imagawa, A. T. Leung, K. P. Campbell, *ibid.*, p. 12309.
23. J. Striessnig *et al.*, *FEBS Lett.* **212**, 247 (1987).
24. P. L. Vaghy *et al.*, *J. Biol. Chem.* **262**, 14337 (1987).
25. V. Flockerzi, H.-J. Oeken, F. Hofmann, *Eur. J. Biochem.* **161**, 217 (1986); M. Sieber, W. Nastainczyk, V. Zubor, W. Wernet, F. Hofmann, *ibid.* **167**, 117 (1987).
26. M. M. Hosey *et al.*, *Biochem. Biophys. Res. Commun.* **147**, 1137 (1987).
27. M. E. Morton and S. C. Froehner, *J. Biol. Chem.* **262**, 11904 (1987).
28. A. T. Leung, T. Imagawa, B. Block, C. Franzini-Armstrong, K. P. Campbell, *ibid.* **263**, 994 (1988).

29. D. Ferry, M. Rombusch, A. Goll, H. Glossmann, *FEBS Lett.* **169**, 112 (1984); J. Galizzi *et al.*, *J. Biol. Chem.* **261**, 1393 (1986).
30. A. Schmid, J. Barhanin, T. Coppola, M. Borsotto, M. Lazdunski, *Biochemistry* **25**, 3492 (1986); M. Takahashi and W. A. Catterall, *Science* **236**, 88 (1987).
31. T. Schneider and F. Hofmann, *Eur. J. Biochem.* **174**, 369 (1988); J. Striessnig, H.-G. Knaus, H. Glossmann, *Biochem. J.* **253**, 39 (1988); D. R. Ferry, A. Goll, H. Glossmann, *ibid.* **243**, 127 (1987).
32. J. Talvenheimo, M. Tamkun, W. A. Catterall, *J. Biol. Chem.* **257**, 11868 (1982); J. Tanaka, J. Eccleston, R. Barchi, *ibid.* **258**, 7519 (1983); J. Weigele and R. Barchi, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 3651 (1982); R. Rosenberg, S. Tomiko, W. Agnew, *ibid.* **81**, 1239 (1984); S. A. Tomiko, R. L. Rosenberg, M. C. Emerick, W. S. Agnew, *Biochemistry* **25**, 2162 (1986).
33. M. Tamkun, J. Talvenheimo, W. Catterall, *J. Biol. Chem.* **259**, 1688 (1984); S. Kraner, J. Tanaka, R. Barchi, *ibid.* **260**, 6341 (1985); D. S. Duch and S. R. Levinson, *J. Membrane Biol.* **98**, 43 (1987).
34. R. Hartshorne, B. Keller, J. Talvenheimo, W. Catterall, M. Montal, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 240 (1985); E. Recio-Pinto, D. S. Duch, S. R. Levinson, B. W. Urban, *J. Gen. Physiol.* **90**, 375 (1987); R. Furman, J. Tanaka, P. Mueller, R. Barchi, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 488 (1986).
35. D. J. Messner and W. A. Catterall, *J. Biol. Chem.* **261**, 211 (1986); D. J. Messner, D. J. Feller, T. Scheuer, W. A. Catterall, *ibid.*, p. 14882 (1986); F. J. Tejedor, E. McHugh, W. A. Catterall, *Biochemistry* **27**, 2389 (1988).
36. B. M. Curtis and W. A. Catterall, *Biochemistry* **25**, 3077 (1986); K. Nunoki, V. Florio, W. A. Catterall, unpublished results.
37. A. Schmid, J. Renaud, M. Lazdunski, *J. Biol. Chem.* **260**, 13041 (1985); J. Arreola, J. Calvo, M. C. Garcia, J. A. Sanchez, *J. Physiol. (London)* **393**, 307 (1987).
38. V. Flockerzi *et al.*, *Nature* **323**, 66 (1986); J. Talvenheimo, J. F. Worley, M. T. Nelson, *Biophys. J.* **52**, 891 (1987); L. Hymel, J. Striessnig, H. Glossmann, H. Schindler, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 4290 (1988).
39. S. R. Levinson, D. S. Duch, B. W. Urban, E. Recio-Pinto, *Ann. N.Y. Acad. Sci.* **479**, 162 (1987).
40. W. M. James and W. S. Agnew, *Biochem. Biophys. Res. Commun.* **148**, 817 (1987).
41. C. Waechter, J. W. Schmidt, W. A. Catterall, *J. Biol. Chem.* **258**, 5117 (1983); D. Bar-Sagi and J. Prives, *J. Cell. Physiol.* **114**, 77 (1983); S. Sherman, J. Chrivia, W. A. Catterall, *J. Neurosci.* **5**, 1570 (1985).
42. E. Recio-Pinto, W. B. Thornhill, D. S. Duch, S. R. Levinson, B. Urban, *Soc. Neurosci. Abst.* **17**, 92 (1987); T. Scheuer, L. McHugh, F. Tejedor, W. A. Catterall, *Biophys. J.* **53**, 541a (1988).
43. H. Windisch and H. A. Tritthart, *J. Mol. Cell. Cardiol.* **14**, 431 (1982); I. Hisatome, T. Kiyosue, S. Imanishi, M. Arita, *ibid.* **17**, 657 (1985); K. Ono, T. Kiyosue, M. Arita, *J. Physiol. (Japan)* **49**, 501 (1988); J. A. Ribiero and A. M. Sebastiao, *Br. J. Pharmacol.* **81**, 277 (1984).
44. M. Noda *et al.*, *Nature* **312**, 121 (1984).
45. M. Noda *et al.*, *ibid.*, p. 320, 188 (1986); T. Kayano, M. Noda, V. Flockerzi, H. Takahashi, S. Numa, *FEBS Lett.* **228**, 188 (1988).
46. V. Auld *et al.*, *J. Gen. Physiol.* **86**, 10a (1985); V. Auld *et al.*, *Neuron* **1**, 449 (1988).
47. T. Tanabe *et al.*, *Nature* **328**, 313 (1987).
47a. S. B. Ellis *et al.*, *Science* **241**, 1661 (1988).
48. H. Guy and P. Seetharamulu, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 508 (1986); H. R. Guy, in *Molecular Biology of Ion Channels: Current Topics in Membrane Transport*, W. Agnew, Ed. (Academic Press, San Diego, CA, in press).
49. W. A. Catterall, *Annu. Rev. Biochem.* **55**, 953 (1986); *Trends Neurosci.* **9**, 7 (1986).
50. R. Greenblatt, Y. Blatt, M. Montal, *FEBS Lett.* **193**, 125 (1985).
51. E. M. Kosower, *ibid.* **182**, 234 (1985).
52. L. Salkoff *et al.*, *Science* **237**, 744 (1987).
53. J. Connor and C. Stevens, *J. Physiol. (London)* **213**, 21 (1971); E. Neher, *J. Gen. Physiol.* **58**, 36 (1971).
54. L. Salkoff and R. Wyman, *Nature* **293**, 228 (1981); C.-F. Wu and F. Haugland, *J. Neurosci.* **10**, 2626 (1985); L. Timpe and L. Y. Jan, *J. Neurosci.* **7**, 1301 (1987); C. K. Solc, W. N. Zagotta, R. W. Aldrich, *Science* **236**, 1094 (1987).
55. M. A. Tanouye, A. Ferrus, S. C. Fujita, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 6548 (1981).
56. D. M. Papazian, T. L. Schwarz, B. L. Tempel, Y. N. Jan, L. Y. Jan, *Science* **237**, 749 (1987); A. Kamb, L. E. Iverson, M. A. Tanouye, *Cell* **50**, 405 (1987); A. Baumann *et al.*, *EMBO J.* **6**, 3419 (1987).
57. T. L. Schwarz, B. L. Tempel, D. M. Papazian, Y. N. Jan, L. Y. Jan, *Nature* **331**, 137 (1988); O. Pongs *et al.*, *EMBO J.* **7**, 1087 (1988); A. Kamb, J. Tseng-Crank, M. A. Tanouye, *Neuron* **1**, 421 (1988).
58. B. L. Tempel, D. M. Papazian, T. L. Schwarz, Y. N. Jan, L. Y. Jan, *Science* **237**, 770 (1987).
59. C. B. Gundersen, R. Miledi, I. Parker, *Nature* **308**, 421 (1984); E. Sigel, *J. Physiol. (London)* **386**, 73 (1987); N. Dascal, T. P. Snutch, H. Lübbert, N. Davidson, H. A. Lester, *Science* **231**, 1147 (1986).
60. K. Sumikawa, I. Parker, R. Miledi, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 7994 (1984); C. Hirono *et al.*, *Brain Res.* **359**, 57 (1985).
61. A. Goldin *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 7503 (1986); D. Krafte *et al.*, *J. Neurosci.* **8**, 2859 (1988).
62. M. Noda *et al.*, *Nature* **322**, 826 (1986); W. Stuhmer, C. Methfessel, B. Sakmann, M. Noda, S. Numa, *Eur. Biophys. J.* **14**, 3831 (1987); H. Suzuki *et al.*, *FEBS Lett.* **288**, 195 (1988).
63. T. Tanabe, K. G. Beam, J. A. Powell, S. Numa, *Nature*, in press.
64. L. C. Timpe *et al.*, *ibid.* **331**, 143 (1988); L. E. Iverson, M. A. Tanouye, H. A. Lester, N. Davidson, B. Rudy, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 5723 (1988).
65. R. M. Stroud and J. Finer-Moore, *Annu. Rev. Cell Biol.* **1**, 317 (1985); H. R. Guy and F. Hucho, *Trends Neurosci.* **10**, 318 (1987); P. N. T. Unwin, G. Zampighi, *Nature* **283**, 545 (1980).
66. S. Oiki, W. Danho, M. Montal, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 2393 (1988).
67. J. Lear, Z. Wasserman, W. DeGrado, *Science* **240**, 1177 (1988); B. Christensen, J. Fink, R. Merrifield, D. Mauzerall, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 5072 (1988).
68. C. Armstrong, *Physiol. Rev.* **61**, 644 (1981); ——— and F. Benzanilla, *J. Gen. Physiol.* **63**, 533 (1974); *ibid.* **70**, 567 (1977).
69. R. Aldrich, D. Corey, C. Stevens, *Nature* **306**, 436 (1976); R. Horn and C. Vandenberg, *J. Gen. Physiol.* **84**, 505 (1984).
70. T. Gonoi and B. Hille, *J. Gen. Physiol.* **89**, 253 (1987).
71. C. M. Armstrong, F. Bezanilla, E. Rojas, *ibid.* **62**, 375 (1973); D. C. Eaton, M. S. Brodwick, G. S. Oxford, B. Rudy, *Nature* **271**, 473 (1978); G. S. Oxford, C. H. Wu, T. Narahashi, *J. Gen. Physiol.* **71**, 227 (1978).
72. D. Gordon, D. Merrick, D. A. Wollner, W. A. Catterall, *Biochemistry* **27**, 7032 (1988); P. Vassilev, T. Scheuer, W. A. Catterall, *Science* **241**, 1658 (1988).
73. H. Meiri *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 5058 (1987).
74. S. Rossie, D. Gordon, W. A. Catterall, *J. Biol. Chem.* **262**, 17530 (1987).
75. F. J. Tejedor and W. A. Catterall, *Proc. Natl. Acad. Sci. U.S.A.*, in press.
76. R. D. Gordon *et al.*, *ibid.* **84**, 308 (1987); R. D. Gordon, Y. Li, W. E. Fieles, D. L. Schotland, R. L. Barchi, *J. Neurosci.*, in press.
77. Supported by research grants from NIH, NSF, the Muscular Dystrophy Association, and Miles Laboratories and by research contract DAMD 17-84-C-4130 from the U.S. Army Research Office.