

# Voltage-gated sodium and potassium channels

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The existence of biological electricity has been known for 200 years. Descriptions of various excitable membranes, and of numerous varieties of proteins involved in membrane excitability, have become increasingly available over the last 50 years. In the last decade, a 'new' molecular approach has been used with much success to study voltage-gated ion channels that form the basis of electrical excitability of nerve and muscle membrane. It is hoped that these methods will help to provide a molecular understanding of the wealth of physiological data that have been accumulated over the past years. This review attempts to summarize current knowledge and progress towards such a goal.

LUIGI Galvani discovered in 1791 that current applied to the sciatic nerve of a frog caused reproducible contraction of the innervated muscle. From this experiment he hypothesized that 'animal electricity' was involved in nerve activity. In about 1850 Matteucci and Du Bois-Reymond first recorded electrical currents in animal tissue—in injured muscle fibre—using newly developed galvanometers. The surface of the muscle fibre was electropositive compared to the injured site; current flowed in the external circuit from the electropositive surface to the injured site. This current diminished during activity. Thus the active site on the muscle fibre became negative relative to the resting surface. Julius Bernstein observed that this electronegativity propagated like a wave down the fibre, with a velocity approximately equal to that determined by Helmholtz for the rate of propagation of the nerve impulse. Since then, nerve impulses have been associated with propagating waves of membrane depolarization<sup>1</sup>.

It was apparent to nineteenth-century physiologists that electric currents in neurons were probably carried by ions. Several ideas and notions about membrane potentials, most significantly those of Walter Nernst, were synthesized in the 'membrane theory' by Bernstein in 1902. It was known that  $K^+$  is at a higher concentration inside the cell than outside. The membrane theory proposed that at rest the excitable membrane is selectively permeable to potassium ions. Thus, the resting potential of the cell is negative, close to the potassium equilibrium potential. Bernstein went on to propose that neuronal stimulation causes a large local increase in membrane permeability to all ions, in a process now referred to as 'membrane breakdown'. The resultant membrane depolarization stimulates adjacent

points of the nerve cell where the same process occurs. Thus a wave of depolarization propagates down the length of the axon.

The conclusions above, on the nature of excitable membrane, were based on a large amount of plausible, circumstantial evidence. Crucial technical advances, such as the use of cathode-ray oscilloscopes for electrophysiological measurements by Erlanger and Gasser, and the invention of the voltage-clamp apparatus by Kenneth Cole and Howard Curtis, allowed more accurate measurements of membrane potentials. Equally crucial was the use of the squid giant axon: it allowed intracellular recordings to be made for the first time, and also the chemical analysis of axoplasm to accurately determine intracellular ionic concentrations. These advances set the stage for decisive experiments conducted between 1937 and 1952 by a small group of researchers, including Alan Hodgkin, Andrew Huxley and Bernard Katz in Great Britain, and Curtis Cole and Howard Curtis in the United States. These experiments transformed the main features of the membrane theory, from plausible hypothesis, to established fact. It became clear that nervous impulses are propagated as electrical signals; that action potentials and synaptic potentials result from changes in membrane permeability to specific ions. The exact mechanisms by which membrane permeability is regulated remained unknown, until much later. These seminal papers have been compiled into a single volume by Cooke and Lipkin<sup>2</sup>, and are briefly reviewed here.

A major departure from the membrane theory stemmed from the discovery that, during an action potential, membrane potential not only ceased to be negative but actually reversed in sign and became positive. This positive 'overshoot' was explained by the sodium theory of Hodgkin and Katz<sup>3</sup>. Hodgkin *et al.* clearly demonstrated that during the action potential, the membrane became briefly selectively permeable to sodium ions and the membrane potential approached the sodium equilibrium potential. Hodgkin and Huxley showed that the permeability to sodium decayed with time in a process they called inactivation. Both the inactivation of sodium conductance and activation of a potassium conductance forced the membrane potential back to the resting value. In the now-famous Hodgkin and Huxley model, they suggested that sodium and potassium permeabilities behave independently; that independent membrane-bound particles, sensitive to

transmembrane potential, behave as gates to control sodium and potassium permeabilities. Although proposed from studies on the squid giant axon, this model satisfactorily explains nerve membrane activity in a variety of organisms.

### Membrane ionic conductances

Membrane potential changes involve the passage of ions into out of cells. We now know that this occurs through membrane proteins called ion channels. While it was clear from Hodgkin and Huxley's experiments that specific mechanisms regulating the conductance of membranes to sodium and potassium ions are present in nerve membrane, the nature of these mechanisms was the subject of much speculation till the mid-sixties. In 1964, tetrodotoxin (TTX), a toxin from puffer fish (later identified in a variety of species, including salamanders and frogs), was shown to block the rise in sodium conductance in lobster giant axons without affecting potassium or leakage conductances<sup>4</sup>. This specific effect on sodium conductance was observed in several preparations, including frog myelinated axons and the squid giant axon. The same effects were observed with an independently isolated, structurally related toxin called saxitoxin (STX). The quaternary ammonium ion tetraethyl ammonium (TEA) was found to specifically block the rectifying potassium conductance in the squid giant axon<sup>5</sup>. Thus the two ionic pathways were clearly separated by these pharmacological agents, in a manner similar to the specific inhibition of enzymes. It was generally accepted from these and other studies that independent ion channels coexist in excitable membrane, a belief compatible with the assumptions made by Hodgkin and Huxley.

Other toxins that bound sodium channels with different affinities in resting and depolarized membrane were identified. The most reasonable interpretation of these results was that sodium channels undergo conformational changes on membrane depolarization exposing or masking toxin binding sites during the process<sup>6,7</sup>. If this were true, it followed that charged groups were present in the structure to sense membrane potential, and that these charges moved in response to membrane depolarization. Currents associated with the movements of the hypothetical gating charges were recorded by Armstrong and Benzanilla<sup>8</sup>. By the early seventies, it became quite clear that ion channels are membrane proteins that undergo conformational changes that enable them to act as ion-selective pores in the membrane. The change in membrane permeability observed in skeletal muscle membrane upon the application of acetylcholine is mediated by ion channels that 'open' in response to acetylcholine binding. The increase in sodium and potassium permeabilities

observed by Hodgkin and Huxley in squid axon is mediated by ion channels that open in response to membrane depolarization. The immense diversity of distinct channel types in even simple nervous systems was, however, not appreciated till much later.

Shortly after Hodgkin and Huxley's pioneering work, microelectrodes began to be used widely for intracellular recordings of action potentials in nerve and muscle from several biological phyla. A wide variety of action potential waveforms and firing patterns were observed. It was believed early on that all electrically excitable cells had sodium and potassium conductances similar to the squid giant axon; that differences observed in various action potential waveforms occurred because of small, species-specific differences in time and voltage dependences of the kinetic parameters of the channels. However, several distinct types of ion channel, with varied kinetic properties, were soon found to be present in the same organism. It is now apparent that the squid giant axon has fewer kinds of channel than almost any other cell type that has been examined; it is clear that action potentials in most excitable cells involve varying contributions from a very large number of different channel types. The identification of all channels contributing to a complex action potential, for example that of a heart Purkinje cell, may be an extremely difficult process.

New channel types continue to be identified, as more excitable cells are analysed with varied pharmacological agents and electrophysiological methods. The 'patch clamp' technique, pioneered by Neher and Sakmann in the late seventies, has allowed the analysis of currents through a single-channel molecule. Using this method it is possible to achieve gigaohm seals between an electrode and a small patch of biological membrane and thus measure  $10^{-12}$  amps of current (current through a 'normal' channel is in the range between 5 and 50 pA). The patch may be detached from the cell in an inside-out or outside-out conformation and examined in a variety of experimentally chosen conditions. This allows a description of channel kinetics on the basis of detailed analysis of currents through single channels; the sophistication of this analysis allows very small differences in physiological properties to be detected. However, very few channels have been examined at this level. In the rest of this review I attempt to describe the development of our current understanding of ion-channel structure, diversity and function. Molecular studies on channel proteins and channel genes have played a vital role in this process.

### Voltage-gated ion channels

Ion channels that open in response to changes in membrane potential are called voltage-gated channels.

They are highly selective for specific ions and are named for the ions that they conduct. Most voltage-gated channels open in response to membrane depolarization although a few, such as the anomalous rectifier, open on membrane hyperpolarization. After opening from the resting state, some channels close in a time-dependent fashion into an inactive state and then take a significant time to recover to the activatable (resting) state. The best example of such channels is the sodium channel from squid giant axon. Non-inactivating channels (for example delayed-rectifier-type potassium channels in squid axon) remain open until membrane repolarization makes them adopt the closed conformation. Figure 1 shows the cycle of a channel from a closed state through open and inactivated states.

Voltage-gated sodium channels, potassium channels, calcium channels and chloride channels have been identified in most multicellular organisms. Each of these categories includes several diverse channel types that vary in a variety of parameters, including voltage sensitivities, rates of opening, open times, conductances, rates of inactivation, rates of recovery from inactivation, sensitivities to  $\text{Ca}^{++}$  and other divalent cations, sensitivities to second messengers and to various pharmacological agents. Many of these specific channel types are found in a wide range of phyla.

## Sodium channels

### Biochemical purification and functional reconstitution

Physiologically characterized sodium channels have many features in common. They have high selectivity for sodium ions over all other physiological cations ( $\text{Li}^+ > \text{Na}^+ > \text{NH}_4^+ > \text{K}^+ > \text{Ca}^{++}$ ) and rates of conductance higher than  $10^7$  ions per molecule per

second<sup>9</sup>. Sodium channels activate rapidly in response to membrane depolarization ( $t_{1/2}$  for sodium conductance rise in the squid axon is approximately 0.5 ms at 10°C) and then inactivate in a time-dependent manner ( $t_{1/2}$  for inactivation is approximately 2 ms). The channels are then insensitive to voltage during a quiescent (inactive) period. The membrane must then be hyperpolarized for many milliseconds before the channels recover their earlier properties. This cycle of activation, inactivation, and recovery from inactivation is observed in most sodium channels. Molecular models of sodium channels strive to explain these basic processes.

The logical first step in the study of the molecular structure of sodium channels was the biochemical purification of the protein. In 1973 the TTX-binding component of the garfish olfactory nerve was successfully solubilized using nonionic detergents. However, the toxin-binding activity of the solubilized preparation was too unstable to be used as an assay for more extensive purification. The addition of phospholipid and calcium to the detergent-solubilized sodium channel was later found to stabilize dramatically the toxin-binding activity of the channel<sup>7</sup>. The STX receptor has now been purified to a high degree of homogeneity from several sources, including electric eel electroplax, several mammalian skeletal muscle and mammalian brain. It is apparent that all sodium channels include a large, heavily glycosylated subunit ( $\alpha$ ) containing the TTX binding site, and in some tissues include one or two smaller subunits. In rat brain the smaller subunits are called  $\beta 1$  and  $\beta 2$ ,  $\beta 2$  is attached to the  $\alpha$ -subunit by disulphide bonds while  $\beta 1$  is noncovalently attached.

Purified tritiated TTX (or tritiated STX) receptors from rat brain, eel electroplax and rat skeletal muscle have been reconstituted into lipid bilayers. Single-channel currents from these reconstituted channels show relatively normal physiological characteristics. The channels are sensitive not only to TTX, but also to unrelated toxins such as veratridine and batrachotoxin that affect native sodium channel in characteristic ways. Thus, the TTX receptor appears to include most of the entire functional sodium channel. An observable difference is that most purified sodium-channel preparations show no activity in lipid bilayers in the absence of  $\alpha$ -bungarotoxin (BTX), but then behave like BTX-modified channels with little inactivation and a low threshold of activation. The reason for this phenomenon is not known. However, similarly purified channels reconstituted into multilamellar lipid vesicles show channel activity in excised patches even in the absence of BTX (ref. 10). The most significant knowledge gained from these studies is that: (i) the purified proteins are indeed identical to the sodium channel, and (ii) the large subunit of the sodium

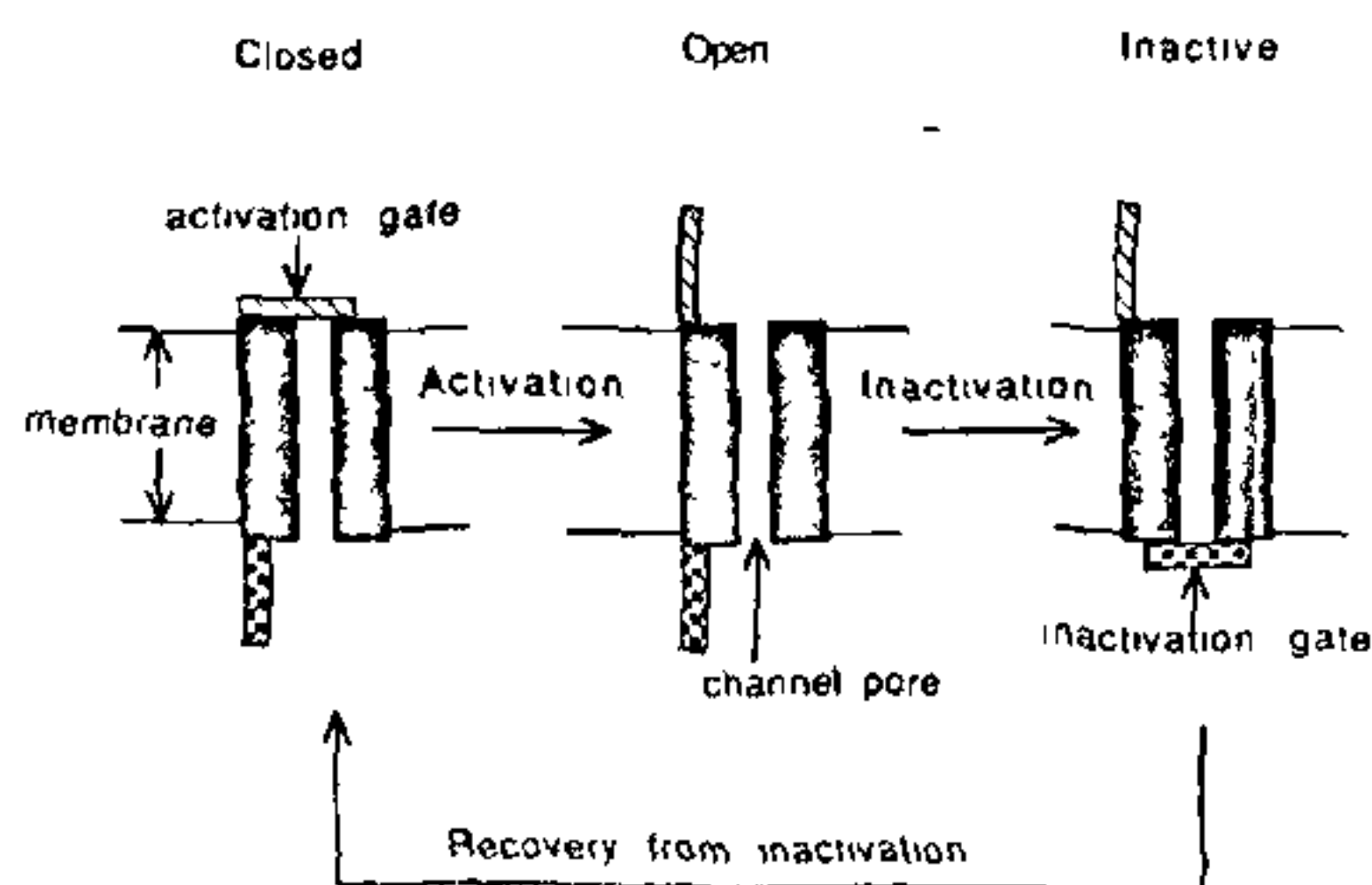


Figure 1. The cycle of a channel through a minimum of three states—a closed state, an open state and an inactivated state. The nature of the conformational changes involved in channel activation, inactivation and recovery from inactivation is not understood. It is likely that several closed, open and inactive states are available to a single channel. Thus the state diagram for a real channel is likely to be much more complicated than this one. See text and ref. 42 for further information.

channel is capable of independently forming a voltage-gated, sodium-selective pore with much of the pharmacological sensitivities displayed by sodium channels in excitable membrane. The successful purification of sodium channels paved the way for molecular cloning of the sodium-channel gene.

### Diversity

Despite popular belief to the contrary, several independent experiments began to suggest that sodium channels were a diverse population of channel molecules. The major lines of evidence were from three sources: (i) pharmacological sensitivities of sodium channels, (ii) immunological reactivities of sodium channels, and (iii) electrophysiological properties of sodium currents in various organisms and excitable tissue.

**Pharmacological and immunological evidence.** TTX was the first means of demarcating two classes of sodium channels. TTX-resistant sodium channels were described<sup>11</sup> in cardiac muscle in 1976. Similar channels were found in several other biological tissues, including denervated vertebrate skeletal muscle and in immature nerve and muscle membrane<sup>12,13</sup>. Other toxins, such as  $\mu$ -conotoxin from *Conus geographus*, were used to distinguish TTX-sensitive channels in brain from those in skeletal muscle<sup>14</sup>. Yet another toxin from scorpion venom preferentially bound sodium channels on muscle membrane while showing little affinity for those in the T-tubular system<sup>15</sup>. Monoclonal antibodies raised against purified sodium channels from skeletal muscle were used to identify three subpopulations of sodium channels in adult rat skeletal muscle<sup>16</sup>. These antibodies distinguished channels in the sarcolemma from channels in the T-tubular membrane. Sodium channels in the T-tubule system of slow-twitch muscle fibres could also be distinguished from those in fast-twitch fibres. This analysis also showed that channel subtypes that could be distinguished by one antibody were not distinguished by others. Thus the subtypes shared some common epitopes, and probably represented distinct, but related, proteins.

**Electrophysiological evidence.** Differences in electrophysiological properties of sodium channels have been detected both by voltage-clamp and by patch-clamp analysis. Unusual sodium channels that differed significantly from the classical Hodgkin and Huxley sodium channel were discovered in many tissues. An aesthetically pleasing example, that also serves to demonstrate a distinct biological function for such sodium channels, is in the giant axon of the worm, *Myxicola*<sup>17,18</sup>. In response to touch, the escape response of *Myxicola* is mediated by an action potential through its giant axon.

However, upon repeated stimulation at frequencies greater than 5 Hz, no escape response is generated by later stimuli. This behaviour, called 'adaptation', is mimicked when a *Myxicola* is held in the hand: 'when first picked up (it) will twitch violently, but after several twitches the worm will lie motionless in the hand'. Voltage-clamp analysis of currents carried by the giant axon showed that sodium channels recover very slowly from inactivation (greater than one second for 90% recovery). At high frequencies of stimulation fewer sodium channels are available to respond to succeeding stimuli and hence the axon is incapable of sustained firing. Thus this unusual sodium channel forms the basis for frequency-dependent adaptation of the giant axon. It has not been shown, however, due to a regrettable lack of interest in *Myxicola* physiology, that *Myxicola* has other classes of sodium channels.

Electrophysiological evidence exists for diverse populations of sodium channels in the same organism. In squid giant axon it has been demonstrated that there are two populations of sodium channels. The major class comprises more than 95% of the sodium channels in the axon and has been described by Hodgkin and Huxley; the minor class called 'threshold channels' activates at potentials close to the resting potential and has a very slow rate of inactivation. Thus these channels dominate behaviour of the axon membrane in the threshold region for action potential initiation<sup>19</sup>. In rat, electrical conductances in several different tissue types have been examined. A noninactivating ( $t_{1/2} > 100$  ms) sodium channel has been identified in the soma of cerebellar Purkinje cells, which have very complex firing patterns. When calcium conductances are blocked, the action-potential waveform in these cells includes a transient sodium-dependent spike probably mediated by Hodgkin-and-Huxley-type sodium channels; this is followed by a smaller, prolonged sodium-dependent plateau, during which a noninactivating sodium channel is active along with various potassium conductances. Both inactivating and noninactivating sodium conductances are TTX-sensitive. The precise contribution of the noninactivating sodium channel to the normal physiology of the cell is not clear; the current is clearly seen only when calcium conductances are blocked<sup>20</sup>.

Single-channel records from rat myotubes and myoblasts show the coexistence of two classes of sodium channel; a TTX-sensitive channel with a 12-pS conductance that activates at more depolarized potentials than a TTX-resistant channel with a smaller conductance (10 pS). The relative proportions of the two channel types change during muscle development. The TTX-resistant channels may serve a similar function in muscle membrane as the threshold channels in squid axon<sup>21</sup>. Three different types of TTX-sensitive sodium currents have been described in membrane

patches from rat heart ventricular cells. These appear to have similar conductances but vary most obviously in their rates of inactivation. Rates of inactivation have been used to classify them as 'fast', 'slow' and 'ultraslow' ( $\tau = 0.6$  ms, 13.9 ms and 130 ms respectively, at  $-30$  mV). It was suggested that these different currents reflected different 'modes' of a given sodium channel molecule rather than different sodium channel subtypes. This issue was not resolved, however, as all the patches used in the studies contained multiple (four to 50) channels<sup>22</sup>.

A different type of sodium channel has also been found in glia. Type-1 astrocytes express a TTX-sensitive sodium channel with slower activation (long latencies to first opening in single-channel records) and slower inactivation (longer mean open times) than neuronal TTX-sensitive channels. In addition, their voltage dependences of activation and inactivation are shifted in a hyperpolarizing direction; this may be an adaptation for glial function as glia have resting potentials more negative than neurons<sup>23</sup>.

Multiple sodium-channel types have been identified in a variety of other preparations. The characterization of these channel types and their physiological functions remain a subject of intense research. A more fundamental problem in the mid-eighties was the mechanism by which these different channel activities were generated. Were they alternative modes of the same protein; if so, how were these modes generated in the cell? Or, were they independent proteins whose synthesis and assembly were under complex spatial and temporal regulation? The other question was one pondered for many years: what is the molecular architecture of the sodium channel?

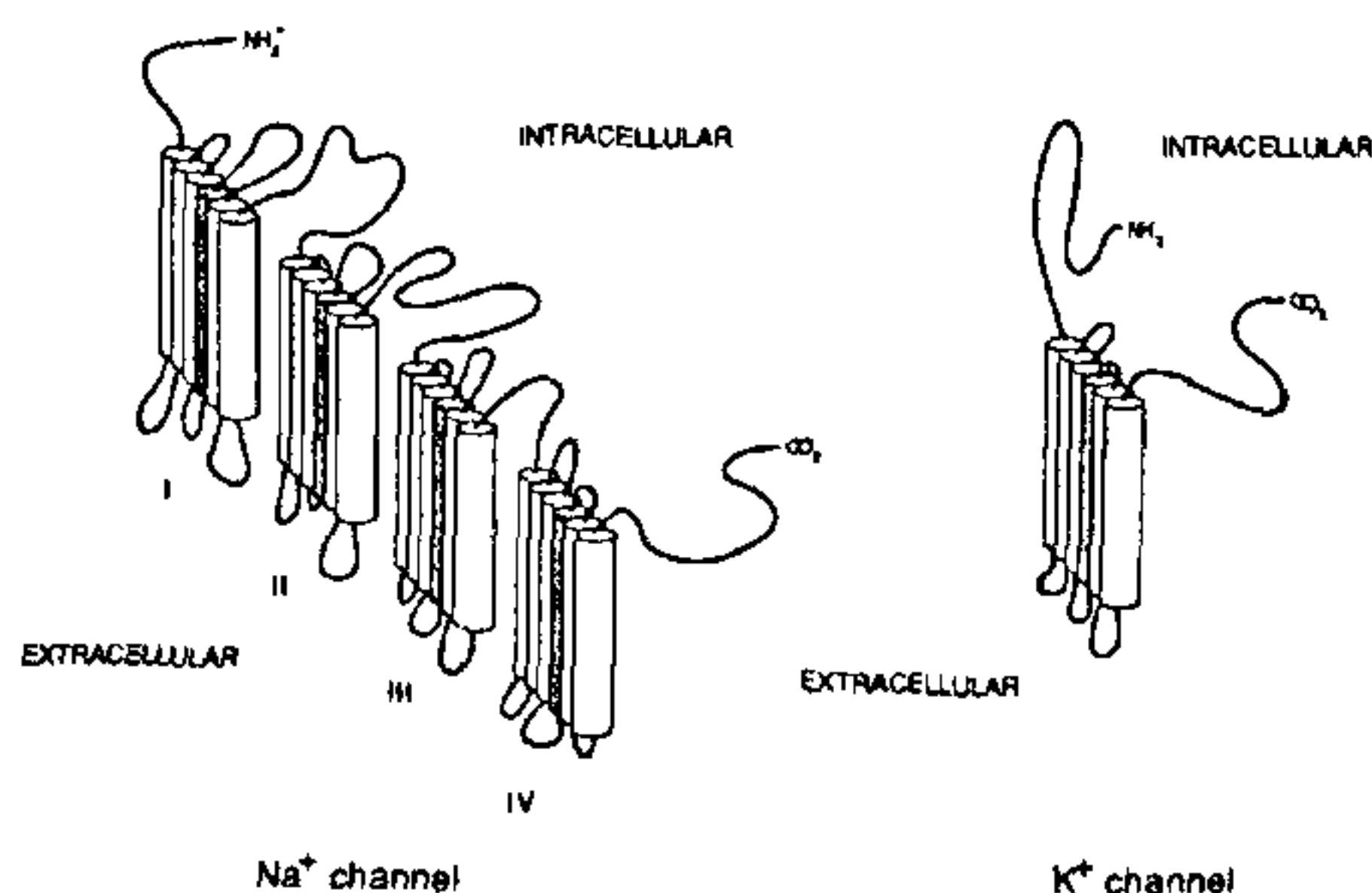
### Molecular genetics

**Sequence of *Electrophorus electricus* sodium channel.** The first sodium channel gene to be cloned was from electric eel electroplax<sup>24</sup>. Protein purified from this tissue was digested with trypsin and the N-terminal sequence of purified tryptic fragments was determined by protein microsequencing. Complementary DNAs from the sodium channel gene were isolated by a combination of immunological screening of an expression library with hybridizations to radiolabelled oligonucleotides corresponding to known peptides. The deduced amino-acid sequence of the channel is briefly described below.

The eel sodium channel is composed of a single large polypeptide 1820 amino acids in length. This corresponds roughly with the molecular weight of the deglycosylated, purified channel. The sequence includes 10 potential sites for N-glycosylation, sufficient for the addition of sugar groups that comprise 29% of the mature protein. The absence of an N-terminal signal sequence suggests that the N-terminus of the polypeptide is intracellular.

The most striking feature of the protein is the presence of four internal repeats that are roughly 50% identical to one another. The repeats are about 250 amino acids in size; they are referred to, in order, as 'homology unit' or 'homology domain' I, II, III and IV. Each homology domain contains six characteristic hydrophobic segments about 24–38 amino acids in length. The segments (S1–S6) are capable of spanning the plasma membrane. Segments S5 and S6 are very hydrophobic and are flanked by charged residues; these characteristics are typical of membrane-spanning segments. S1, S2 and S3 generally contain a few charged residues. Negatively charged groups predominate in S1 and S3 while both positive and negative charges are found in S2. If an  $\alpha$ -helical structure is assumed for these segments, the charged side chains cluster on one face of the helix. Thus each of these segments is capable of forming an amphipathic helix in which the polar residues are shielded from lipid membrane. The S4 segment in each repeat is positively charged with 4–8 arginine or lysine residues; while these basic amino acids are located at every third position, the other residues in S4 are hydrophobic. Thus, if one assumes a  $3_{10}$ -helical conformation, all the positive charges line up on one face of the helix. In an  $\alpha$ -helical conformation the charged groups are arranged in a spiral; in a  $\beta$ -pleated sheet they extend alternately to opposite sides of the peptide backbone. Negatively charged residues are found between S5 and S6 of all repeats, as also in the region between homology domains II and III.

These features of the eel sodium-channel sequence clearly suggest a rough structural organization for the channel (see Figure 2). The individual homology



**Figure 2.** Proposed transmembrane topologies of voltage-gated sodium and potassium channels. The shaded segments correspond to positively charged segment 4 (S4) of each homology domain that are likely to serve as membrane-potential sensors (see text). Early papers on potassium channels suggest a different topology, with an extra segment (H5) spanning the membrane. This H5 segment is shown as part of the extracellular loop between S5 and S6. The topology of cloned voltage-gated calcium channels is identical to that of the sodium channel shown; however, the sizes of the cytoplasmic and extracellular loops vary<sup>104</sup>.

domains are arranged pseudosymmetrically in the channel; thus there are an even number of transmembrane segments (four or six) per homology domain. The unusual positively charged S4 segments may sense and respond to changes in membrane potential<sup>24</sup>. Several detailed models for sodium-channel structure were spawned from the sequence of the eel sodium channel<sup>25-28</sup>. There is, however, a paucity of data to confirm or refute details of the models; for this reason, I only discuss features of the models that are supported by experimental data.

Structural models for sodium channels were refined by the isolation and characterization of a family of closely related rodent sodium-channel genes. Noda *et al.*<sup>29</sup> used eel sodium-channel cDNA probes to isolate and characterize three distinct, sodium-channel genes expressed in rat brain (RSC1, RSC2 and RSC3). The sequence of a fourth sodium-channel gene specifically expressed in skeletal muscle, SKM1, has recently been reported<sup>30</sup>. An independently isolated rat brain sodium-channel gene, RatIIA, shows more than 99% DNA sequence identity with RSC2; it appears likely that it is the same gene as RSC2 isolated from a different strain of rats<sup>31</sup>.

The basic structural organization of the rat sodium channels is identical to that of the eel channel; each has four homology domains with similar characteristic features. The amino-acid sequences of the channels are extremely similar to one another. The overall homology (per cent sequence identity) among the rat channels is about 75%; this figure exceeds 90% within the four homology domains. Significant differences are present in regions linking the internal repeats to each other. Some features, such as the clustered negative charges between homology domains II and III, present in the eel sequence, are absent in the rat sodium channels; they probably do not serve key functions assigned to them in some models<sup>24,25</sup>. Additionally, the rat brain channels have a large insertion of about 170 residues with multiple consensus sites for phosphorylation by A-kinases between homology domains I and II; this insertion is absent in the eel channel and also in SKM1. Significantly, a short, lysine-rich linker region between domains III and IV is remarkably well conserved among all known sodium-channel sequences. Two sodium-channel genes, DSC and *para*, have been cloned and characterized in *Drosophila*. These studies show that sodium channels in invertebrates are similar in sequence to those in vertebrates, and that invertebrates may also have diverse sodium-channel subtypes<sup>32-34</sup>.

## Physiological properties of cloned sodium channels

One of the most important requirements for making structure-function correlations in a protein is the ability

to study the activity of the protein once its sequence has been established. As the eel sodium-channel gene could not easily be engineered to express functional protein, RSC2 was the first sodium-channel gene to be successfully expressed in a heterologous expression system<sup>35</sup>. In this commonly used method, *in vitro*-transcribed mRNA was microinjected into stage V-stage VI *Xenopus* oocytes. A two-microelectrode voltage clamp was used to monitor channel activity in the oocyte membrane two days after the injection. In these assays, RSC2 mRNA induced voltage-dependent sodium currents that activated and inactivated rapidly; the currents were abolished by 1  $\mu$ M TTX but were insensitive to  $\mu$ -conotoxin. This experiment served several purposes: (i) it showed that the large subunit of the channel could independently form sodium channels in *Xenopus* oocytes, (ii) it showed a method for the detailed characterization of an isolated channel type, (iii) it also provided a method for the study of mutant channels modified *in vitro*, a procedure critical for correlating channel structure with its function. Although studying a channel in a heterologous environment is not ideal, the oocyte expression system has been extremely useful not only for characterization of cloned sodium channels<sup>30,35,36</sup> but also for studying channels using RNA from specific tissues, and for cloning new channel genes<sup>37-39</sup>.

RSC3, RatIIA and SKM1 channels have also been successfully expressed in *Xenopus* oocytes. No significant differences have been reported between RSC2 and RSC3 currents<sup>40</sup>. Though the first report of the physiological properties of RatIIA indicated a significant difference from RSC2 in the voltage dependence of activation, this difference was later attributed to an inadvertent cloning artefact that altered a critical leucine residue in the channel<sup>31,41</sup>. Thus no real physiological differences have yet been detected between functionally expressed brain sodium channels. The SKM1 current shows a dramatic difference in pharmacology; it is insensitive to 100 nM TTX and is completely blocked by 5 nM  $\mu$ -conotoxin.

Voltage-sensitive sodium currents may be induced in *Xenopus* oocytes by the injection of total mRNA prepared from rat brain. These currents inactivate more rapidly than currents induced by the injection of *in vitro* transcribed mRNA (cRNA) from cloned channels. Both currents may be made indistinguishable if small-molecular-weight RNA (2-4 kb), fractionated from total brain, is coinjected into the oocytes with cRNA. This observation suggests a role in inactivation for small proteins, presumably the small subunits of rat brain sodium channels<sup>36</sup>.

## Structure-activity correlations

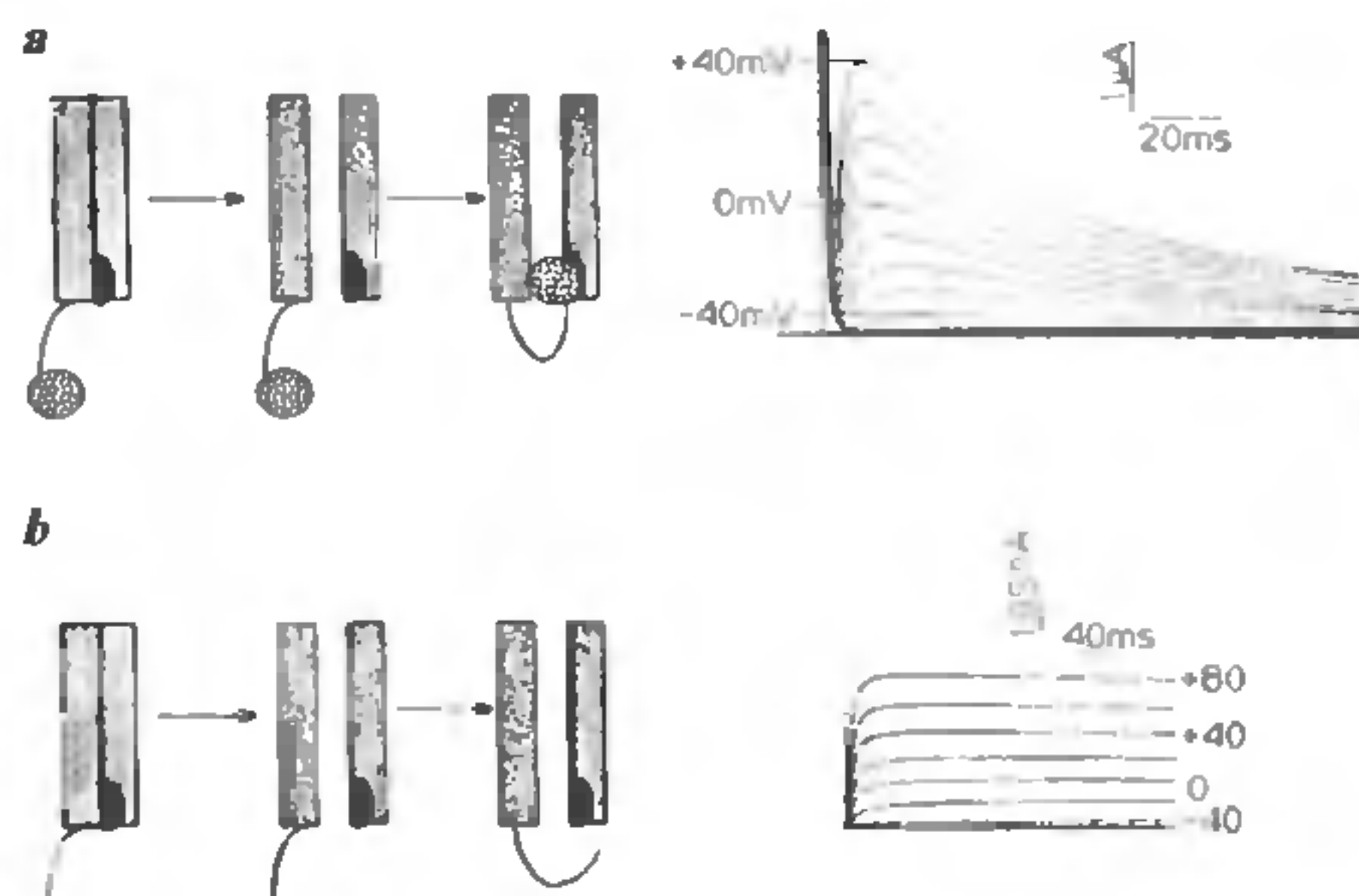
There is a surfeit of models of hypothetical sodium-

channel structures and of correlations of modelled structures with specific channel functions<sup>28,42,43</sup>. I shall not discuss these models here. Instead, I briefly review the sparse experimental data that exist in this area of research. Most of these data associate specific amino-acid residues or specific regions in the protein with particular channel functions such as activation or inactivation.

**Activation and inactivation.** It has been apparent for several years that voltage-gated sodium channels contain charged residues located close to or within the membrane. Depolarization of membrane could cause this 'activation gate' to move, triggering conformational changes in the protein<sup>42,44</sup>. The positively charged S4 segment of the sodium channel has all the features expected of a voltage sensor; all popular models suggest that S4 resides in the membrane and associates with negatively charged groups in other transmembrane segments. To test the role of S4 as a voltage sensor, a series of mutant sodium channel cDNAs were constructed *in vitro*; they carried one or more sequence alterations in the S4 segment of the first homology domain. Channels expressed from these mutant cDNAs were assayed in *Xenopus* oocytes for altered physiological function. A decrease in the steepness of the potential dependence of activation was observed with decrease in positive charge on the S4 segment. Such a result is predicted by mathematical models of voltage-dependent activation gates. The experiment has been interpreted as a confirmation of the hypothesis that S4 is the voltage sensor in voltage-gated channels and that positive charges in S4 constitute (at least part of) the gating charge in sodium channels<sup>45</sup>.

The intracellular perfusion of squid giant axon with various proteases effectively removes the inactivation process of the sodium channel. This was first reported with pronase, a mixture of several distinct proteinases with broad specificity<sup>8,46</sup>. The experiment was repeated with proteases purified from crude pronase, and also with other pure preparations of proteases such as trypsin, papain, ficin and  $\alpha$ -chymotrypsin. The effectiveness of the different enzymes in blocking inactivation was correlated with the enzyme specificities. Based on this analysis, it was proposed that an intracellular peptide including lysine or arginine residues was likely to be involved in inactivation<sup>47</sup>. The mechanism of inactivation was suggested to involve blocking of the channel pore by a sequence (a 'ball' at the end of a peptide 'chain') that was cleaved off by pronase. In this 'ball-and-chain model' for inactivation, the pore binding site became accessible to the 'ball' only when the channel opened (Figure 3).

The cloning of sodium-channel genes allowed the identification of the pronase-sensitive sequences. A lysine-rich region containing potentially protease-sensitive sites has been found between homology domains III and IV of



**Figure 3.** A summary of the main features of the ball-and-chain model for channel inactivation and the experimental support for this model; the data shown are for an inactivating human voltage-gated potassium channel called huKII (M. K. Mathew, M. Ramaswami and M. A. Tanouye, unpublished data). **a.** A wild-type channel protein: the channel opens revealing a binding site for the 'ball', the ball binds to the site near the mouth of the channel pore and physically blocks the channel. Thus, one observes time-dependent inactivation. The panel on the right shows real data from a voltage clamp of huKII currents in a microinjected *Xenopus* oocyte. Potassium current is shown on the y axis, and time on the x axis. Each trace corresponds to a different transmembrane potential. Thus voltage-dependent huKII potassium currents are first activated at a transmembrane potential of about -50 mV. At a constant transmembrane potential, inactivation can be seen as a time-dependent decay of current. Thus at +40 mV huKII has a  $t_{1/2}$  of inactivation of approximately 40 ms. **b.** These diagrams represent a mutant huKII channel protein in which a large piece of the N-terminal cytoplasmic loop has been cleaved off. The deletion has little effect on the efficiency of channel assembly; however, the mutant channel does not inactivate over a period of one second (Mathew *et al.*, data not shown). This result fits well with the ball-and-chain model for inactivation, in which the deleted sequences contain the 'ball'.

all cloned sodium channels. Antibodies to this peptide were generated and were perfused into myoballs (colchicine-treated skeletal muscle cells) that were analysed under voltage clamp. The antibody was found to specifically reduce the rate and extent of channel inactivation<sup>48</sup>. In parallel experiments, sodium-channel cDNA was cleaved between homology domains III and IV. RNA transcribed *in vitro* from the two cDNA fragments were coinjected into *Xenopus* oocytes. Functional but non-inactivating sodium channels were expressed on oocyte membrane. These experiments, to identify sodium-channel sequences involved in inactivation, have all pointed toward the same conserved intracellular segment.

However, other sequences are also likely to be involved, as judged by the effect of some extracellularly applied ligands, including antipeptide antibodies and peptide neurotoxins from scorpion, sea anemone, coral and snail. These ligands affect inactivation in different ways<sup>49</sup>. There is little known about the molecular mechanism of channel inactivation. Indeed, I do not know of an enzyme with a function truly analogous to inactivation. Autoregulation of enzymes such as many

protein kinases, or subunit dissociation of G-proteins on activation, are the closest analogies to this process. Some revealing experiments that address the mechanism of inactivation of potassium channels are mentioned later in this review.

**The channel pore.** Sequences close to the sodium-channel pore have been identified in an aesthetically pleasing fashion. It has been known for a long time that TTX-sensitive channels may be made insensitive by treatment with carbodiimides or trimethyloxonium, reagents that modify carboxylic acid groups. This process concurrently reduces single-channel conductance<sup>9</sup>. Thus the TTX binding site is likely to be intimately associated with the conducting pore. Point mutants have been constructed in the rat sodium channel RSC2 that alter specific acidic residues on presumed extracellular regions of the sodium channel. One of these changes, glutamic acid 387 to glutamine, causes a greater-than-1000-fold reduction in binding affinity for TTX. The same change also reduces the unitary conductance of the sodium channel. This acidic residue lies between S5 and S6 of homology domain II (ref. 50). This region has been proposed to form the channel pore in at least one structural model<sup>27</sup>.

The most impressive feature in sodium-channel function is its high selectivity for sodium combined with a conduction rate close to that expected for free diffusion of sodium ions. Mutant channels that affect ion-selectivity have not yet been constructed; no convincing structural model for this function exists to direct the mutagenesis experiments. In some experiments specific, amphipathic peptide fragments from the sodium channel have been reconstituted into lipid bilayers, and their ability to assemble into channels has been assayed. Ion channels with several different conducting states, presumably due to different-sized aggregates of the amphipathic peptides, have been detected in these experiments. It appears clear from these studies that any sequence capable of forming an amphipathic helix can also form very weakly ion-selective channels. Twentytwo-amino-acid peptides from either S3 or S4 can independently form ion channels in lipid bilayers<sup>51,52</sup>. The former form voltage-insensitive, cation-selective channels with no selectivity for sodium over potassium; the latter form voltage-gated, and, surprisingly, cation-selective channels with no preference for sodium over potassium. These experiments, while interesting in their own right, do not determine the real sodium channel pore lining. There is no reason to assume that the peptide sequences that line the pore interact directly with lipid membrane.

### Current research

Several groups have been involved in identifying new

sodium channel genes using low-stringency hybridization and polymerase chain reaction (PCR) techniques on cDNA from different tissues. At least eight different sodium channel  $\alpha$ -subunit genes from rat have been partially characterized in several laboratories. One of these is reportedly specific to cardiac muscle. The cellular or subcellular distributions, physiological properties and genetic map positions of these genes are subjects of active research. A large collaborative effort to identify other sodium channel subunits is in progress; this has not yet been successful. A few groups have been involved in studying transcriptional regulation and post-translational modification of sodium channels; processes that could conceivably serve to modulate, *in vivo*, cellular responses to stimulation (discussed in greater detail in the next section on potassium channels). Many groups have been studying the structure of sodium channels with several different methods and objectives. Detailed study of sodium-channel structure is completely justified by the general paucity of structural information on membrane proteins. The more commercial purpose of this study is the development of sodium channel subtype-specific drugs and the prospect of 'channel engineering'<sup>53</sup>.

Genetic methods are being used to identify and study genes involved in invertebrate sodium-channel function. These genetic experiments, confined to *Drosophila*, are reviewed in refs. 54–56.

### Potassium channels

Potassium-selective channels are probably the first ion-selective channels to have evolved and are ubiquitously found in all phyla that have been examined. They have had more time to diversify than other channel types and, consequently, have been recruited to serve several diverse physiological functions in different cell types. Specific blends of potassium-channel types in cell membrane largely determine the various action-potential waveforms and neuron-specific firing patterns that have been observed in complex nervous systems<sup>42,57,58</sup>. Potassium channels may be categorized according to their physiological properties into four groups that may partially overlap: (i) voltage-gated potassium channels, (ii) inward rectifiers, (iii)  $\text{Ca}^{++}$ -activated potassium channels, (iv) neurotransmitter- and second messenger-regulated potassium channels. Only voltage-gated potassium channels are discussed here; the other potassium-channel groups are mentioned briefly in refs. 57, 59.

#### Voltage-gated potassium channels

**Nomenclature.** Voltage-gated potassium channels have been roughly grouped into two categories: the 'delayed rectifier' (K) channels and the 'A' channels. The prototypic delayed rectifier is that described by

Hodgkin and Huxley in the squid giant axon. The potassium current in squid axon activates with a delay on membrane depolarization, and reaches its peak more slowly than the voltage-dependent sodium current. The current inactivates relatively slowly ( $\tau > 500$  ms) at potentials greater than the threshold for action-potential generation; it is also relatively insensitive to intracellular calcium concentration. All similar potassium currents responsible for action potential repolarization are called delayed rectifiers or K-type currents.

The prototypic A current was first described in the soma of a molluscan bursting neuron. The outward potassium current in this cell can be separated under voltage clamp into two components with clearly distinguishable properties. One component is similar to the classical delayed rectifier; the other activates rapidly at subthreshold potentials. The subthreshold current inactivates rapidly with a steep dependence on membrane potential; the current is almost completely inactivated by voltage prepulses near the action-potential threshold. In addition, the inactivating current is more sensitive to 4AP and less sensitive to TEA than the K current. These physiological properties, coupled with a lack of dependence on intracellular calcium, are the characteristics that define A channels. A-type channels dominate the behaviour of the neuron at subthreshold potentials; in bursting neurons, they control the interspike interval<sup>60</sup>. The cellular functions assigned here to A-type and delayed-rectifier channels are often shared by other channel types, most significantly by calcium-activated potassium channels.

*Physiological diversity.* Since Connor and Stevens's analysis in 1971, several voltage-gated potassium channels have been physiologically characterized from a variety of excitable tissue; many of these have properties intermediate between the classical delayed-rectifier and A-type channels. As there is often no clear distinction between these two groups of ion channel, very similar channels in different preparations have been labelled as either A type or K type by different investigators<sup>57</sup>. Thus voltage-gated potassium channels may traverse the entire spectrum from classical A-type to classical delayed-rectifier-type channels. It is possible that the present classification into A-type and K-type channels is a historical legacy that currently gives us little insight into the properties and functions of potassium channels.

Several subtypes of voltage-gated potassium channels have been identified in the same organism using voltage-clamp and patch-clamp techniques in the manner described for sodium channels. Voltage-clamp analysis of the frog node of Ranvier has revealed three distinct voltage-gated potassium currents: a low-threshold, fast-transient current (f1), a higher-threshold fast transient (f2) and a slowly activating non-inactivating component(s)<sup>61</sup>. These observations have been partially supported by fluctuation

analysis of potassium conductances in large patches from the node of Ranvier. However the latter study has been interpreted as evidence for multiple conducting states of the same channel<sup>62</sup>; this interpretation is hard to reconcile with the clear differences in sensitivities to peptide toxins between f1, f2 and s channels reported by Dubois<sup>61</sup>. The macroscopic delayed-rectifier current changes during maturation of *Xenopus* spinal neurons. The developmental regulation of two populations of voltage-gated potassium channels with different unitary conductances underlies this phenomenon. It has been reported that, in addition to the increase during maturation of the density of both channel types, the kinetics of the larger-conductance type also changes; the molecular basis for this phenomenon is not known<sup>63</sup>. Instead of an overwhelming, comprehensive discussion of different voltage-gated potassium channels, it probably suffices to hint at the diversity of these channels by listing the characteristics of voltage-gated potassium channels in rat—the organism best studied by cellular physiologists. Table 1 (which draws extensively from Rudy<sup>57</sup>) shows the various channel types and their characteristics.

Several problems are apparent in trying to gauge the full extent of potassium-channel diversity from independent characterizations of potassium currents in various cell types: (i) The membranes of different cells have different compositions. It has been shown that lipid composition can affect both the unitary conductance of channels, and their activation and inactivation properties<sup>57,64</sup>. It has also been shown that channels may associate with cytoskeletal elements in some tissues and such association could have effects on physiological properties<sup>65</sup>. (ii) Different procedures and protocols have been used by different groups. For example, pharmacological experiments performed by Dubois on the node of Ranvier potassium channels were not repeated by Conti *et al.* in their later study of the same system, or by Standen *et al.* in their study on frog skeletal muscle channels. (iii) It is possible that the same channel type has multiple functional states that may vary widely depending on factors such as temperature or surrounding medium<sup>62</sup>. In this scenario, diversity of functional channels does not really represent molecular diversity of channel proteins. (iv) Macroscopic potassium currents are not easily resolved into separate components. Thus potassium channel components analysed by voltage clamp may easily be mixtures of similar channels that are later resolved by more sophisticated methods. However, even in the face of these technical problems, it is clear that voltage-gated potassium channels are a diverse family of proteins that vary widely in their voltage dependencies, conductances and pharmacological sensitivities. Potassium channels may also be modulated differently by second-messenger systems, and this modulation might add to the observed diversity of voltage-gated channels.

Table 1. Voltage-gated potassium channels in rat (Adapted from Rudy<sup>57</sup>)

Preparation	Activation Threshold (mV)	Kinetics (ms)	Inactivation rate $k_{0.5}$ (ms mV <sup>-1</sup> )	4AP block	TEA block external	Others	Other properties/ references
Sympathetic neurons	1) < -40 (25 C) 2) -60	10-20 (26 mv, 25 C) v. fast	~5000 55'-75	NB $K_d > 1$ mM	3 mM		114, 115, 116
Hippocampal Pyramidal neurons	~ -40	~50 (20 C)	2000-4000 (20 C)	NB 5 mM	$K_d \sim 10$ mM		117
Skeletal muscle 20 C	~ -50	1)6 2)60	200-300 1500-3000	Block NB	Block 40 mM Block >>40 mM		118 Slow I in slow-twitch muscle
Spermatogenic cells	~ -40	~20 (22 C; -10mV)			Block 126 mM		119
Cultured hippocampal neuron	-60	3-10	10-40/-75	$K_d \sim 2$ mM			117,120
Cardiac ventricular cells	-40	2-10	20-40/-60	$K_d \sim 2$ mM			121
Locus coeruleus neurons	-60	-	~100/-50(100%)	$K_d \sim 1$ mM			122
Cultured sensory neurons	-50	-	30-50/<-65	-			123
CA1 hippocampal cells	-60	very fast	50%,-60	-		1 mM DTX	124
Sensory neurons nodose ganglion	-60	-	1000-2000	Block 30 $\mu$ M		DTX	125
PC12 cells	Kx)0 Kw)-20 Ky)-60 Kz)-55	50 10(0 mV) Variable 2.5(10 mV)	very slow/-120 40-80/-70 Very slow 100-500/-70	No Yes No Yes(?)	Block Yes Yes Yes		126, 127; $g = 5-9$ pS 126, 127; $g = 14-18$ pS 126, 127; $g = 5-9$ pS 126, 127; $g = 11-14$ pS

**Pharmacology and biochemistry.** The biochemistry of potassium channels is still in its infancy. Until very recently, there were no specific high-affinity ligands (with  $K_d$  values in the nanomolar range) that could be used for the purification of voltage-gated potassium channels. In the last few years, several naturally occurring peptide toxins that bind voltage-gated potassium channels have been characterized. These include dendrotoxin (DTX) and  $\beta$ -bungarotoxin ( $\beta$ -BTX) from snake venom; mast cell degranulating peptide (MCDP) from bee venom; and noxious toxin (NTX) (and possibly charybdotoxin, CTX) from scorpion venom. Several other crude preparations of venom from a variety of sources have also shown effective blockage of voltage-gated potassium currents. These toxins have been used more for characterization of physiologically identified potassium currents than for biochemical purification of potassium channels. In general, each of these toxins blocks several different voltage-gated channels that have a wide range of physiological properties: for example, DTX blocks a low-threshold, fast-transient potassium current in rat hippocampus, in addition to a slowly activating, non-inactivating current in guinea pig dorsal root ganglion and an unusual, fast-activating, non-inactivating current in rat visceral afferent neurons<sup>66</sup>. This pharmacological evidence suggests that voltage-gated potassium channels may be a family of structurally related proteins. Toxins

that bind non-voltage-gated potassium channels have been much better characterized. Several drugs used in the treatment of cardiovascular disorders, and those used in the treatment of a form of diabetes act upon potassium channels; biochemical studies of these channels is a very active area of research that is out of the scope of this review<sup>66-68</sup>.

Dendrotoxin receptors from brain have been biochemically purified. While it remains possible that this is a heterogeneous mixture of polypeptides, a single polypeptide of 76-80 kDa has been shown to contain DTX, MCDP and  $\beta$ -BTX binding sites; specific binding of <sup>125</sup>I-labelled MCDP and <sup>125</sup>I-DTX is inhibited by  $\beta$ -bungarotoxin. A polypeptide of 38 kDa copurified in this procedure. It is believed that the potassium channel is a multimer of these subunits with yet unspecified stoichiometry<sup>69</sup>.

### Molecular genetics

**Primary structure.** An elegant combination of genetic, electrophysiological and molecular studies on the *Shaker* locus of *Drosophila melanogaster* led to the first cloning of a voltage-gated potassium channel gene. Several distinct mRNAs are generated by alternative splicing at the *Shaker* (*Sh*) locus. At least six of them are capable of directing the synthesis of potassium channels when independently microinjected into *Xeno-*

*pus* oocytes; the nature of these different currents is discussed later. The mRNAs follow a simple pattern, in which variable 5' and 3' ends are spliced onto a central 'constant' region. The constant region of functional Shaker polypeptides is about 400 amino-acid residues in size; the variable amino domains range from 31 to 61 residues; the variable carboxyl domains are between 170 and 240 residues. Several features of the sequence of Sh channels are discussed below<sup>70-72</sup>.

The four functional amino domains of the different Sh channels show no similarities to one another or to other known sequences; there are no obvious distinctive features in these sequences. The two carboxyl regions (that have been shown associated with functional channels) begin at a putative transmembrane segment (H6/S6). There is considerable homology between the two regions until a little after this last putative transmembrane segment; after a conserved glutamine-rich region, the sequence diverges completely until the last three amino-acid residues (Thr-Asp-Val). The molecular significance of these sequences is yet unclear.

The constant region shows many more distinctive features. It has five (or, in some models, six) putative transmembrane segments that are clustered within about 250 amino-acids. The fourth segment (S4) resembles the putative voltage sensor of the sodium channel, in containing several (seven) iterations of the motif Arg/Lys-X-Y (where X and Y are hydrophobic amino acids). Thus there are compelling similarities between the Shaker constant region and one homology domain of a voltage-gated sodium channel. Five iterations of a leucine heptad (a leucine residue at every seventh position) occur adjacent to and downstream of the S4 segment<sup>73</sup>. Such leucine-heptad repeats have been shown to be involved in subunit association and protein-protein interactions in a family of DNA-binding proteins<sup>74</sup>. Less striking, but distinct, leucine-heptad repeats are also found at a similar location in voltage-gated sodium- and calcium-channel sequences.

Due to the several observed sequence similarities between voltage-gated potassium and sodium channels, topological and structural models for potassium channels have drawn heavily from models for sodium channels. It is believed that voltage-gated potassium channels are a multimeric (probably tetrameric) assembly of smaller polypeptides analogous to single homology domains of the sodium channel (Figure 2). The idea that potassium channels are a multimeric assembly of subunits is supported by genetic data from *Drosophila*<sup>75-77</sup>.

Several homologous genes that encode potassium-channel subunits have been recently cloned from a variety of species, including *Drosophila*<sup>78</sup>, rodents<sup>79-90</sup> and man<sup>91,92</sup>. The functional and phylogenetic relationships between these various channels have been discussed earlier<sup>82,90,92,93</sup>.

### *Functional properties of Shaker channels in oocytes*

Eight Shaker cDNAs have been individually expressed in *Xenopus* oocytes. These experiments have served several functions. Most importantly, they establish that a single Shaker polypeptide may assemble into a functional potassium channel, at least in *Xenopus* oocytes; thus the *Shaker* locus may encode at least eight homomultimeric channels. The properties of the different currents indicate specific functions that reside both in the constant domain and in the different amino and carboxyl domains<sup>94-97</sup>. All Shaker channels are identical in potassium conductance, selectivity, voltage dependence of activation and inactivation, and in sensitivity to 4AP and CTX. These functions presumably reside in the constant region—in sequences common to all Sh channels. However, cDNAs derived from the *Shaker* locus encode potassium channels that vary widely in their inactivation properties. When all combinations of amino and carboxyl domains are analysed, it appears that the amino domains determine the rate of inactivation of the channels; the carboxyl domains appear to be primarily responsible for the rate of recovery from inactivation. Significantly, both fast-inactivating, transient potassium channels, and very slowly inactivating, delayed-rectifier-type potassium channels arise from the *Shaker* locus. This suggests that, in general, the two types of channel may be evolutionarily and structurally related. This is in accordance with the pharmacological evidence and the physiological descriptions of different potassium channels discussed in the earlier sections.

Eight distinct, homomultimeric channels are generated by alternative splicing at the *Shaker* locus. An exciting possibility is the formation of heteromultimeric channels, each with different properties. This would result in an almost absurdly large number of potassium channels generated by subunit shuffling. It has been shown that coinjection of two Shaker mRNAs (ShA-ShB from *Drosophila*) into *Xenopus* oocytes results in the formation of channels with kinetic properties intermediate between the parental channel types<sup>98</sup>. Thus, it appears that at least some heteromultimeric Shaker potassium channels may be formed in a cell expressing two different Sh mRNAs. Similarly, it has also been shown that distinct heteromultimeric rat or human potassium channels form in *Xenopus* oocytes coinjected with pairs of cRNAs encoding different potassium-channel subunits<sup>91,99,100</sup>; the *in vivo* significance of this finding is intriguing, but unclear<sup>100</sup>.

The *in vivo* significance of channel properties studied in any heterologous system may, of course, be debated. The reservations about these data are especially justified because differences have been observed between cloned channels expressed in different cell types. *Drosophila* Shaker channels in *Xenopus* oocytes are

CTX-sensitive, while in transgenic *Drosophila* muscle (in which the wild-type *Shaker* gene has been deleted), the channels are CTX insensitive; the reason for this dramatic difference is not known but it may result from different post-translational modifications, or from other, as yet unidentified, potassium-channel subunits present in one of the cell types<sup>101,102</sup>. Rat sodium channels (RatIIA) expressed in frog oocytes differ from the channels expressed in (CHO) Chinese hamster ovary cells—this difference has been ascribed to the availability, in the hamster cells, of small sodium-channel subunits<sup>103</sup>.

### Structural models for Shaker channels

Voltage-gated potassium channels were cloned and sequenced in 1987; their similarities to voltage-gated sodium channels were immediately obvious. At about the same time, the sequence of a voltage-gated calcium channel was reported<sup>104</sup>. The calcium-channel sequence was very similar to the sodium channel, both in the overall organization into homology domains, and in the organization within the homology domains. Thus the molecular evidence suggests that these voltage-gated channels belong to an evolutionarily related family; this was predicted by Hille<sup>42</sup> on the basis of a systematic analysis of channel properties and their distributions through different phyla. Although there are no published structural models for the potassium- and calcium-channel proteins, much of the current thinking about their structures derives from models for the sodium channel. Thus voltage-gated potassium channels are thought to be tetramers of small subunits, each subunit analogous to a sodium-channel homology domain. The activation gate is presumed to be S4, and mutagenesis experiments of positive charges in S4 confirm that voltage-dependent activation is altered by mutations in this region<sup>105</sup>.

Other sequences also play a role in voltage-dependent activation. One of these is an interesting sequence motif found in potassium channels, and fairly conserved in sodium channels. This 'leucine zipper' motif (a leucine-heptad repeat) occurs shortly after S4 in the channel sequence<sup>73</sup>. Alterations of single leucine residues, to other hydrophobic residues such as valine, cause profound (up to 100 mV) shifts in the voltage dependence of activation, and also change the slope of the macroscopic conductance-versus-voltage curves<sup>106</sup>. Similar results have been seen in the sodium channel where one leucine residue in zipper motif was inadvertently altered<sup>41</sup>. While this indicates a role for the leucine residues in voltage-dependent behaviour, the exact role of the leucine repeats is far from established.

The amino domains have been associated with channel inactivation by different criteria. First, it appears from an analysis of different Shaker cDNAs,

that the N-terminal 'variable' domains determine the rate of inactivation of the potassium channel. Second, a 20-amino-acid sequence, near the amino-terminus of Shaker potassium channels, is necessary for channel inactivation. Deleting these residues from a rapidly inactivating Shaker channel converts the channel to an essentially non-inactivating delayed rectifier<sup>107,108</sup>. This result is analogous to the effect of intracellular pronase on the inactivation of voltage-gated sodium channels. However, in a striking experiment, it has been shown that free 20-amino-acid peptide applied to the intracellular surface of the mutant, non-inactivating Shaker channel blocks the open channel in a manner similar to channel inactivation<sup>107,108</sup>. The amino-terminal region of huKII, a human potassium channel, also contains the determinant for channel inactivation; all of these data fit with the 'ball and chain' model for channel inactivation (which would probably be better named as the 'ball, chain and socket' model) (see Figure 3). There are however many unanswered questions about potassium-channel inactivation. These include: (i) the determinants of rates of inactivation—the rate of inactivation does not correlate with either the length of the 'chain', or the affinity of the 'ball-peptide' for the socket; (ii) the identity of the socket; and (iii) the mechanism of recovery from inactivation.

Several mutagenesis studies have identified the likely pore-forming domain of Shaker potassium channels. Charybdotoxin blocks a calcium-activated potassium channel by binding at a site close to the extracellular mouth of the channel pore; it also blocks Sh channels, presumably in a similar fashion. A glutamate residue between S5 and S6 has been implicated in channel association with the toxin<sup>109,110</sup>. This residue, suggested to be at the mouth of the channel pore, is analogous to the glutamate residue in sodium channels implicated in TTX binding<sup>50</sup>. Other experiments have also pointed to the S5–S6 loop as the pore-forming domain of the channel. In the most striking experiment, chimaeras were constructed between two homologous rodent potassium channels (NGK2 and drk1) that had different unitary conductances. It was shown that a chimaeric drk1 channel, with 21 amino acids from the S5 to S6 loop substituted by the homologous segment in NGK2, showed conductance properties of NGK2. Thus the S5–S6 segment contains major determinants of the actual conducting pore<sup>111</sup>. Other experiments have shown that single-amino-acid substitutions in the S5–S6 domain of Shaker potassium channels change the unitary conductance of the channel<sup>112</sup>; some such mutations alter the relative selectivity of the channel for ammonium ions with respect to sodium and potassium ions<sup>113</sup>. From all of these criteria it appears that the S5–S6 loop functions as the channel pore. The structure of the pore is completely unknown; for a model see ref. 28.

## Current research

Several groups have been using molecular, biochemical and electrophysiological methods to study potassium-channel structure. Voltage-gated potassium channels, because of their smaller size, offer some technical advantages over sodium channels. The eventual goals of these efforts are a basic understanding of channel structure, and the more commercial possibilities of channel engineering and drug design.

Several laboratories continue to isolate and characterize other voltage-gated potassium channel genes from both vertebrates and invertebrates. The main goals of these studies are a comprehensive description of voltage-gated potassium-channel diversity, and an effective correlation of cloned channels with those implicated in specific physiological functions. While there has been considerable progress on this front over the last three years, it remains an active area of research.

The molecular biology of potassium channels has hitherto lagged behind studies on sodium channels and acetylcholine receptors. The main reason for this has been the absence of high-affinity ligands to purify and characterize the channel proteins. These ligands are currently being developed at a fast pace; at least three of these—DTX (which binds a class of voltage-gated potassium channels), and CTX and apamin (which bind nonoverlapping classes of calcium-activated potassium channels with high affinity)—are now being used for the purification of potassium channels. Our current molecular knowledge of potassium channels derives ultimately from elegant genetic studies that are feasible only in *Drosophila*, among the higher eukaryotes. These genetic methods are currently being used to characterize and isolate new genes involved in potassium-channel function; other non-Shaker-related families of potassium channels could conceivably be discovered by these methods.

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