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Mechanisms of sodium channel inactivation

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Rapid inactivation of sodium channels is crucial for the normal electrical activity of excitable cells. There are many different types of inactivation, including fast, slow and ultra-slow, and each of these can be modulated by cellular factors or accessory subunits. Fast inactivation occurs by a 'hinged lid' mechanism in which an inactivating particle occludes the pore, whereas slow inactivation is most likely to involve a rearrangement of the channel pore. Subtle defects in either inactivation process can lead to debilitating human diseases, including periodic paralyses in muscle, ventricular fibrillation and long QT syndrome (delayed cardiac repolarization) in the heart, and epilepsy in the CNS.

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Abbreviations

A	alanine
C	cysteine
F	phenylalanine
GEFS+	generalized epilepsy with febrile seizures plus
H	histidine
I	isoleucine
IFMT	isoleucine, phenylalanine, methionine and threonine
M	methionine
Q	glutamine
T	threonine

Introduction

Voltage-gated sodium channels play a crucial role in regulating the electrical excitability of animal cells, being primarily responsible for the depolarization phase of the action potential. The channel consists of a highly processed α subunit that is approximately 260 kDa, and is associated with one or more accessory subunits (β1, β2 and β3) in certain tissues [1]. The α subunit consists of four homologous domains termed I-IV, each domain contains six transmembrane segments termed S1-S6 and between S5 and S6 there is a hairpin-like P-loop that comprises part of the channel pore (Figure 1). The accessory β2 subunit is covalently linked to the α subunit by disulfide bonds, and the β1 and β3 subunits are noncovalently attached and expressed in a complementary fashion, so that α subunits are associated with either β1 or β3.

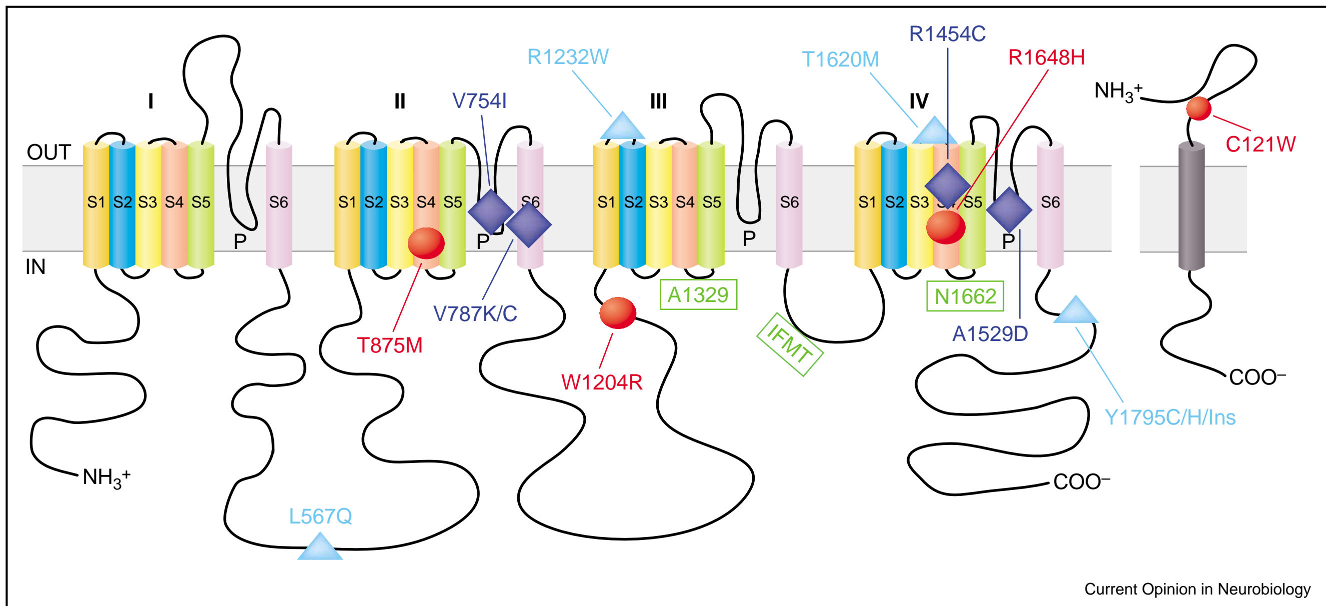
The fundamental properties that enable sodium channels to carry out their physiological roles include rapid, voltage-dependent activation, which opens the channel, and inactivation. Inactivation closes the channel and prevents it from reopening until there has been sufficient time for recovery, which helps to determine the frequency of action potential firing. In addition, inactivation makes the cell refractory to firing during a long depolarization, thus preventing a breakdown of ionic gradients and cell death. Many toxins (including batrachotoxin, scorpion toxins and sea anemone toxins), insecticides (pyrethroids), and clinically useful drugs (local anesthetics, anti-arrhythmics and anti-convulsants) affect sodium channel inactivation.

Although inactivation is often referred to as a single process, there are at least two distinct kinetic classes of inactivation, termed fast and slow. In addition, there may be two types of fast inactivation, the conventional process involving an inactivating particle that is part of the channel blocking the pore, and a second type in which open channels are blocked by an extrinsic particle [2••,3•]. Inactivation is made even more complex by the fact that both fast and slow inactivation are modulated by multiple factors, including the cell type [4]. This review concentrates on recent advances in four areas of sodium channel inactivation research. The first part summarizes studies that use synthetic peptides to understand the molecular mechanism of fast inactivation. The second section discusses progress in identifying the regions of the sodium channel that are involved in slow inactivation. The third part summarizes the methods by which fast inactivation is modulated, particularly by the carboxy-terminus and β subunits. The final section discusses the clinical effects of aberrant inactivation, and concentrates on the effects of CNS sodium channel mutations that cause epilepsy.

Fast inactivation

Fast inactivation in voltage-gated sodium channels occurs by a 'ball-and-chain' or 'hinged lid' mechanism, in which a cytoplasmic region (the inactivating particle) occludes the pore by binding to a region nearby (the docking site). The inactivating particle consists of a portion of the cytoplasmic linker connecting domains III and IV, with the crucial region centering on a four amino acid stretch consisting of isoleucine, phenylalanine, methionine and threonine (IFMT) (Figure 1). The docking site consists

Figure 1



Schematic diagram of the sodium channel α and β subunits. The relative lengths of the cytoplasmic linkers represent those found in $Na_v1.2$. The green rectangles indicate the amino acids important for fast inactivation, specifically the crucial IFMT motif in the inactivating particle and the docking site residues A1329 and N1662. The dark blue diamonds represent the mutations in $Na_v1.4$ that affect slow inactivation (V754I, V787K/C, R1454C and A1529D). The light blue triangles indicate the mutations in $Na_v1.5$ that cause long QT or Brugada syndrome (L567Q, R1232W and Y1795C/H/insertion). The red circles represent the mutations that cause GEFS+ (C121W in the in the β1 subunit and T875M, W1204R and R1648H in the $Na_v1.1$ α subunit).

of multiple regions including the cytoplasmic linkers connecting segments 4 and 5 (S4-S5) in domains III and IV and the cytoplasmic end of the S6 segment in domain IV.

Several recent studies have examined the characteristics of these regions by synthesizing isolated peptides and determining their crystal structures. Rohl *et al.* [5] first described the structure of the inactivation particle on the basis of a 53 amino acid peptide that comprised the entire III-IV domain linker. They concluded that the IFMT residues are essential components of a latch that has both hydrophobic and hydrophilic characteristics, with phenylalanine (F) and threonine (T) positioned to directly interact with the docking site. Three studies that examined shorter peptides with various mutations have confirmed and extended those findings. Studies with peptides of 17 [6] or 36 residues [7] showed that the T actively participates in the inactivation process, and that substitution of glutamine (Q) for F separates isoleucine (I) and T, hindering the formation of a hydrogen bond between them. Substitution of methionine (M) for T in the 17mer peptide prevented that residue from acting as a proton acceptor and forming a hydrogen bond, whereas an I to Q substitution did not affect the formation of the bond [8]. The distance between the I and T residues correlated with the ability to inactivate the channel.

With respect to the docking site, Miyamoto *et al.* [9] examined the structure of the S4-S5 linkers in domains III (14mer) and IV (19mer). Their data confirmed results from cysteine mutagenesis experiments suggesting that the IVth domain S4-S5 is α-helical [10,11]. They proposed that outward movement of the voltage sensors (the S4 segments) exposes hydrophobic clusters in S4-S5 that can interact with the inactivating particle, after which the phenylalanine at position 1489 (F1489) interacts with the alanine at position 1329 (A1329) in III S4-S5 [12] and asparagine at position 1662 (N1662) in IV S4-S5 (Figure 1; [13]). This hypothesis is consistent with the theoretical models of Sirota *et al.* [14•], who proposed that a hairpin motif optimizes the interaction between IFMT and its docking site, and that movement occurs around a previously identified hinge that is comprised of glycine (G) and proline (P) residues [15].

Slow inactivation

Slow inactivation is a separate process that does not involve the III-IV linker inactivation particle. One hypothesis to explain slow inactivation is that it results from a structural rearrangement of the pore, similar to the mechanism for C-type inactivation in potassium channels [16]. However, the data that concern this hypothesis are conflicting. Ong *et al.* [17] demonstrated that long depolarizations that resulted in slow inactivation decreased the

accessibility of an engineered cysteine residue in the pore region of domain III, consistent with a rearrangement of the pore. In contrast, Struyk and Cannon [18•] observed no changes in the modification rates of engineered cysteine residues in the pore regions of the four domains after slow inactivation, indicating that the cysteines were equally accessible to the modification reagent before and after slow inactivation. These results suggest that the mouth of the pore does not close during slow inactivation.

The pore may be the site of a conformational change during slow inactivation, but the process involves many other regions of the channel, including IV S4 [19], II S5-S6 [20•] and II S6 (Figure 1; [21]). Modification of an engineered cysteine near the middle of IV S4 in $Na_v1.4$ (R1454C in $Na_v1.4$) enhances slow inactivation [19]. The higher probability of slow inactivation in $Na_v1.4$ compared to $Na_v1.5$ is caused by a single amino acid difference in II S5-S6, the residues V754 in $Na_v1.4$ and I in $Na_v1.5$ [20•]. A single residue in II S6 can alter the kinetics of slow inactivation in $Na_v1.4$, for example, substitution of lysine (K) for V787 enhances the process, whereas substitution of cysteine (C) for V787 retards it [21]. It is likely that slow inactivation involves a significant conformational change of the channel that includes a rearrangement of the pore, but the actual mechanism that prevents ionic flow is still unknown.

Sodium channel inactivation is not limited to two kinetic processes. An even slower process termed ultra-slow inactivation has been observed in $Na_v1.4$ when the alanine at position 1529 (A1529) is replaced by aspartate (D) in the IVth domain P-loop [22]. Although ultra-slow inactivation is also distinct from fast inactivation, entry into the ultra-slow inactivated state is inhibited by binding of the fast inactivation particle, possibly because of allosteric modulation of the outer vestibule [23•], which demonstrates that there are interactions among the different inactivation events.

Modulation by the carboxy-terminus

Although fast inactivation is mediated by the III-IV linker inactivating particle, it can be modulated by the carboxy-terminus of the channel, as demonstrated by several studies that involve $Na_v1.5$. Fast inactivation is slower in $Na_v1.5$ when compared to $Na_v1.4$, and this difference in kinetics is attributable to the first 100 amino acids in the carboxy-terminal region [24]. Similarly, fast inactivation in $Na_v1.5$ is slower than that of $Na_v1.2$, and this difference is also due to the carboxy-terminal region [25]. These results are consistent with the fact that mutations in the carboxy-terminus of $Na_v1.5$ that cause long QT syndrome disrupt fast inactivation [26,27]. Cormier *et al.* [28••] used theoretical modeling and circular dichroism measurements to identify six α-helical segments in the proximal half of the carboxy-terminus of $Na_v1.5$ (there were none in the distal half). Deletion of the distal half of the carboxy-terminus did not affect activation or inactivation of $Na_V1.5$, but a deletion starting with the sixth helical segment (which is highly charged) in the proximal half caused a marked increase in sustained current. They proposed that electrostatic interactions involving the sixth helix in the carboxy-terminus modulate the interaction of the fast inactivation particle with its docking site [28••].

Modulation by the β subunits

Interactions with the β subunits can also modulate fast inactivation, and the effects and the mechanisms that underlie these interactions are dependent on the specific α and β subunits involved. For example, the membrane anchor plus either the intracellular or the extracellular region of β1 is required to accelerate recovery from inactivation of $Na_v1.5$ [29], whereas the extracellular region of β1 is necessary to accelerate inactivation of $Na_v1.2$ [30–32]. The β3 subunit, which has been identified only recently, affects multiple α subunits. It acts similarly to β1 in that it increases the percentage of fast mode gating in *Xenopus* oocytes for $Na_v1.2$, $Na_v1.4$ [33] and $Na_v1.3$ [34], but it is unique in that it increases persistent current through $Na_v1.2$ in tsA-201 cells, which are derived from human embryonic kidney cells [35••]. Neither β3 nor β1 accelerates inactivation of $Na_v1.3$ in Chinese hamster ovary cells, although both subunits shift the voltage-dependence of inactivation in the negative direction and slow down the rate of recovery from inactivation [36]. The β3 subunit also accelerates recovery from inactivation of $Na_v1.5$ cardiac channels in oocytes, which may be physiologically significant because β3 is expressed in the ventricles and Purkinje fibers of the heart [37]. Taken together, all of these results demonstrate that β subunit modulation of sodium channel function is dependent on both the α subunit isoform and the cell type used for expression.

The most physiologically relevant way to determine the role of the β subunits is by construction of knockout mice, which has been accomplished for the β2 subunit by Chen *et al.* [38••]. The β2 knockout mice demonstrate reduced sodium channel density, as determined electrophysiologically and by saxitoxin binding, which results in an increased threshold for action potential generation. The mice display an increased susceptibility to seizures, although they do not demonstrate any other neurological abnormalities. These results support the hypothesis that the presence of the β2 subunit is important for expression of the sodium channel in the neuronal cell membrane [39].

Clinical effects of abnormal inactivation

Sodium channel mutations cause human diseases of skeletal muscle, cardiac muscle and the CNS, and most of these mutations alter some aspect of channel inactivation. Mutations in $Na_v1.4$ cause periodic paralysis, paramyotonia congenita and the potassium-aggravated myotonias,

all of which involve delayed muscle relaxation [40]. Mutations in $Na_v1.5$ cause long QT type-3, which predisposes to ventricular tachycardia (torsades de pointes), and Brugada syndrome, which is manifested as ventricular fibrillation [41]. Mutations in CNS sodium channels cause several types of epilepsy, including generalized epilepsy with febrile seizures plus (GEFS+) [42,43].

It has been proposed that long QT syndrome results from mutations that cause a gain of sodium channel function, whereas Brugada syndrome mutations reduce sodium channel function [41]. Although generally correct, this hypothesis cannot explain the effects of all of the cardiac sodium channel mutations. For example, idiopathic ventricular fibrillation can be caused by either decreased or increased sodium channel inactivation. Destabilization of inactivation (accelerated recovery and slower onset) results from mutations in IIIrd domain S1-S2 (R1232W) and IVth domain S3-S4 (T1620M) [44], whereas acceleration of inactivation results from a mutation in the linker between domains I and II (L567Q) (Figure 1; [45]). In addition, altering a single residue can have opposite effects. Substitution of histidine (H) for Y1795 in the carboxy-terminus accelerates inactivation, whereas substitution of C for Y1795 slows inactivation [46]. The same mutation can even have opposite effects, with insertion of D after position 1795 both increasing sodium channel function by disrupting fast inactivation and decreasing function by augmenting slow inactivation [47].

In the CNS, one mutation that causes GEFS+1 has been identified in the *SCN1B* gene that encodes the β1 subunit [42,48], and 10 mutations that cause GEFS+2 have been identified in the *SCN1A* gene that encodes the $Na_v1.1$ α subunit (*SCN1A*) [43,49–53]. Severe myoclonic epilepsy in infancy, the most severe form of GEFS+, results from a haploinsufficiency (functional loss of one allele) of *SCN1A* [54,55], and mutations that cause GEFS+ have been identified in the *SCN2A* gene that encodes the $Na_v1.2$ α subunit [50].

The β1 subunit mutation that causes GEFS+1 is C121W, which is located in the extracellular immunoglobulin domain (Figure 1). The mutant β1 subunit does not modulate the inactivation properties of $Na_v1.2$ or $Na_v1.4$ as effectively as the wild-type β1 subunit [42,56,57]. Meadows *et al.* [58•] examined the effects of the mutant β1 subunit on $Na_v1.2$ and $Na_v1.3$ channels, and showed that it increases channel availability at hyperpolarized potentials, reduces channel rundown during high frequency activity, and results in a loss of ability to mediate protein–protein interactions that are crucial for channel localization. These effects are consistent with a clinical phenotype of hyperexcitability.

The effects of three of the $Na_v1.1$ mutations have been analyzed, with different results for each mutation (Figure 1). In oocytes, the T875M residue in IInd domain S4 enhances slow inactivation of rat $Na_v1.1$ [59•] and rat $Na_v1.4$ [60], the W1204R residue in the II-IIIrd domain linker shifts the voltage-dependence of activation and inactivation of rat $Na_v1.1$ in the negative direction [61], and the R1648H residue in IVth domain S4 dramatically accelerates recovery from inactivation of rat $Na_v1.1$ [59•] and rat $Na_v1.4$ [62]. The R1648H mutation also causes a marked increase in persistent current through human $Na_v1.1$ in tsA-201 cells, with a slight increase in persistent current for the T875M and W1204R mutations [63•]. These results suggest that different alterations in sodium channel function can lead to a similar seizure phenotype. Increased sodium channel activity that results from accelerated recovery from inactivation or a larger persistent current leads to seizures, presumably by causing hyperexcitability. This is consistent with the finding that limbic seizures and behavioral abnormalities were observed in transgenic mice expressing $Na_v1.2$ channels with an increased persistent current [64•]. It may seem surprising that decreased sodium channel activity resulting from enhanced slow inactivation also causes epilepsy. However, this result is consistent with the effects of mutations in the skeletal muscle $Na_v1.4$ channel that cause periodic paralysis by enhancing slow or fast inactivation [65–67], and the fact that most of the mutations that cause severe myoclonic epilepsy in infancy represent a loss of function from one *SCN1A* allele. Taken together, these results demonstrate that an alteration in the balance of CNS sodium channel activity can lead to epilepsy.

Conclusions

Inactivation is a fundamental property of sodium channels that is crucially important, with subtle defects in either fast or slow inactivation having substantial effects on the physiology of the organism. Therefore, understanding the mechanisms underlying inactivation has important therapeutic implications. The hinged lid model for fast inactivation is well supported by the data, and it is likely that future studies will more clearly define the specific molecular interactions that are involved in the process. On the other hand, the mechanisms underlying slow inactivation or modulation by the carboxy-terminus and β subunits are not understood. As many local anesthetic, anti-arrhythmic and anti-convulsant drugs modulate sodium channel inactivation, it is likely that a better understanding of these processes will make it possible to identify or design drugs that are more specific and effective in treating disorders of sodium channel abnormalities.

Acknowledgements

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