Review

Structure–function studies on the voltage-gated sodium channel

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Recent research on structure–function relationships aspects of voltage-gated sodium channels (VGSCs) are reviewed. Data issued from the literature are summarized and compared, including results from our own studies. The latter deal with the effects of drug binding, deglycosylation and the role of hydrophobic residues in the voltage sensors. Methods mainly consist of circular dichroism (CD) to asses the channel's secondary structure and conductance measurements after reconstitution into planar lipid bilayers. Molecular modelling was also used to tentatively explain experimental data. Since 30% of the channel's mass are glycoconjugates, the effects of removing them were first investigated. Then, the effects of the neurotoxin Batrachotoxin and the anticonvulsant Lamotrigine were studied. Both drugs induced a significant increase in the channel's helical content and a molecular model shows that lamotrigine interacts with residues previously identified as forming the binding sites in the pore. Finally, the role of hydrophobic residues with long sidechains in the voltage sensors (S4s) was investigated. Recent research on related studies on VGSCs are discussed.

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1. Introduction

Voltage-gated sodium channels (VGSC) undergo conformational changes when excitable cell membranes are depolarized. Their α-subunits are organized in four homologous domains, each of which being composed of six transmembrane segments (S1–S6). The pore region is formed by the S5 and S6 segments and their connecting linker—the P loop [1,2]. Previous attempts to decipher the structure of VGSC, especially cryo-electron microscopy of purified channels from the electric eel resulted in relatively low resolution (19 Å) images [3,4]. The high-resolution structure (comparable to the one obtain with various crystallized potassium channels) is still unknown because of major difficulties to crystallize VGSC. However, inferences can be drawn from voltage-gated potassium channels (composed of four identical and independent subunits) whose high-resolution structure has been solved through X-ray crystallography from 1998 onwards [5–8]. More recent structural data obtained by the R. MacKinnon's group include the crystal structure of a prokaryotic potassium channel (Kir3.1) chimera [9], and the atomic structure of a voltage-dependent K⁺ channel in a lipid membrane-like environment [10].

It is well known for some years that one common feature of all voltage-gated ion channels are voltage-sensors represented by transmembrane segments S4s with positively charged residues (arginines and lysines) occurring every three amino acids and separated by two non-polar residues. This motif identifies the four S4 segments as critical components of the voltage-sensors and the first experimental evidence for their role came from charge neutralizations leading to a reduction in gating currents, alteration of channel conductance and kinetics, as well as shifts of the steady-state activation curve [11]. In addition, cysteine scanning mutagenesis experiments showed that S4 segments move their positive charges when excitable cell membranes are depolarized. Their α-subunits are organized in four homologous domains, each of which being composed of six transmembrane segments (S1–S6). The pore region is formed by the S5 and S6 segments and their connecting linker—the P loop [1,2]. Previous attempts to decipher the structure of VGSC, especially cryo-electron microscopy of purified channels from the electric eel resulted in relatively low resolution (19 Å) images [3,4]. The high-resolution structure (comparable to the one obtain with various crystallized potassium channels) is still unknown because of major difficulties to crystallize VGSC. However, inferences can be drawn from voltage-gated potassium channels (composed of four identical and independent subunits) whose high-resolution structure has been solved through X-ray crystallography from 1998 onwards [5–8]. More recent structural data obtained by the R. MacKinnon's group include the crystal structure of a prokaryotic potassium channel (Kir3.1) chimera [9], and the atomic structure of a voltage-dependent K⁺ channel in a lipid membrane-like environment [10].

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and rotated counterclockwise by 37° along its main axis [18]. Finally, several naturally occurring mutations mainly located in S4 segments lead to channel dysfunction and severe diseases ("channelopathies") such as paramyotonia congenita (see e.g. [19]) and hypokalemic paralysis [20].

2. Secondary structure and function of purified sodium channels: effects of deglycosylation and drug binding

Since sodium channels still resist attempts to crystallization by contrast to many potassium channels, circular dichroism (CD) spectroscopy which needs only small amounts of purified channels was used to assess secondary structure. In addition, synchrotron radiation circular dichroism (SRCD) was also used since it extends the spectrum to lower wavelengths (typically 180-200 nm) disclosing complementary features in the secondary structures [21]. Electrophysiology of purified channels reconstituted into planar lipid bilayers from the electric eel *Electrophorus electricus* was implemented to first check the functional integrity of purified channels and test the effects of deglycosylation and drug binding [21,22]. Finally, molecular modeling was also used to visualize sites affected by the two above-mentioned channel modifications.

Approximately 30% of the mass of the sodium channel is carbohydrate, present as glycoconjugate chains mostly composed of N-acetylhexosamines and sialic acid [23,24]. The effects of removing them on the functional and structural properties of highly purified channels were investigated. After enzymatic deglycosylation, channels were reconstituted into planar lipid bilayers. In the presence of batrachotoxin, an alkaloid which remove fast inactivation and thus results in steady-state activation facilitating the recording of single-channel events, the conductance of deglycosylated channels were slightly reduced as compared to that of native channels (13 vs. 18 pS) also in the presence of BTX. In addition, conductance substates (8–9 pS) were observed only with deglycosylated channels (Fig. 1). These electrophysiological modifications are consistent with electrostatic effects due to the reduction of negative electrical charges (present in carbohydrates moieties, especially sialic acids) at the extracellular vestibule of the channel. Synchrotron radiation circular dichroism (SRCD) demonstrated that unlike most carbohydrates, the type of sugars found in VGSC produce a significant dichroic signal in the far-UV region of the spectrum (Fig. 2). This can account for all the measured SRCD-detected differences between native (α-helical and β-contents: 55% and 19%, respectively) and deglycosylated channels (slightly reduced helical content). Thus, no net secondary structural

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**Fig. 1.** Single-channel activity of native (a) and deglycosylated (b) voltage-gated sodium channels purified from the electric eel *E. electricus* and BTX-treated in planar lipid bilayers. The holding potential was −60 mV and bilayers were depolarized to −10 mV. Openings are downward deflections. (c) and (d) are conductance amplitude histograms for native and deglycosylated channels, respectively. From Cronin et al. [21].

**Fig. 2.** Synchrotron radiation circular dichroism spectra of native (in blue) and deglycosylated (in red) channels (a). (b) shows the spectra obtained with colominic acid (in red) and chitopentose sugars (in bleu), whereas (c) shows difference spectra of native minus deglycosylated channels (in green) and net equivalent sugars (in purple). (from Cronin et al. [21]).
changes of the channel protein itself results from deglycosylation of the channel. In conclusion, while the sodium channel’s carbohydrates are not essential for functional and structural integrity, they have a modulating effect on the conductance properties [21]. It should be mentioned here that a previous study found a significantly higher helical content (65%) for sodium channels purified from rat brain and reconstituted into phosphatidylcholine vesicles [25].

The anticonvulsant or antiepileptic drug Lamotrigine (LTG) (for review, see [26]) specifically blocks VGSC, preferentially in the inactivated state through binding to the pore [27]. LTG blocks the channel in a use/frequency-dependent manner and was found to block sustained repetitive firing in mouse spinal chord neurons and hence it is used in the treatment of seizures [28]. On the other hand, Batrachotoxin (BTX), a lipid-soluble alkaloid initially found in amphibian skin [29] but also, more recently and curiously, in the feathers of some birds of New Guinea [30], binds to the inner pore of the sodium channel resulting in steady-state activation, through removing fast inactivation [31,32]. The molecular determinants of both drugs (LTG and BTX) are overlapping but distinct: mutations L1465A and I1469A decreased affinity for inactivated sodium channels up to 8-fold for LTG [27]. Using circular dichroism spectroscopy on purified sodium channels from *E. electricus*, the secondary structure of the channel at rest and in the absence of these ligands was compared with stabilized states in their presence. As the channel shifts to the open state, there is a significantly increased α-helical content, mainly involving the S6 segments, the III-IV linker and the P-loops that form the pore and selectivity filter. A molecular model of the sodium channel has been constructed, based on its homology with bacterial potassium channels whose three-dimensional high-resolution structure has been determined by X-ray crystallography [6]. Automated docking of LTG crystal structure [33] with this channel model produces a structure (Fig. 3) in which the close contacts of the drug are with residues previously identified by mutational studies as forming the binding sites for Lamotrigine [27]. As for the treatment of epilepsy in the central nervous system, recent patents describe new sodium channel blockers with improved characteristics and fewer adverse effects [34,35].

3. Role of branched sidechain hydrophobic residues in the voltage sensors

It was suggested that substituting hydrophobic residues with branched sidechains (such as leucines and isoleucines) for achiral ones or alanines in peptides and proteins change their dielectric properties, especially by cancelling their ferroelectric behavior [36]. This was predicted to translate into the loss of voltage-dependence for ion channels [37].

We first checked and confirmed this hypothesis applying the “peptide approach” which consists in synthesizing peptides mimicking S4 segments substituting key residues such as proline (helix breaker), lysines and arginines and study both conformation by CD spectroscopy and conductance measurements in planar lipid bilayers. This approach had previously been used, especially for the voltage sensors [38,39]. Three peptides were synthesized by the Merrifield solid-phase method [40]. Their aminoacid sequences encompasses S4 of domain III (previously found the most voltage-dependent peptide in another study with peptides mimicking all four S4s of the electric eel sodium channel [41]).

In one peptide, both I and L (italics underlined above) at positions 9 and 15, respectively, were replaced by alanine. The voltage-sensitivity of macroscopic current developed in planar lipid bilayers interacting with this peptide was greatly reduced but this was not the case if only Leu15 was substituted with alanine (Fig. 4). Thus, position 9 appears critical [42]. This isoleucine is near the second arginine at the C-end of the peptide which was found as the most important of all charged residues of S4 for the gating in previous studies on the whole channel [43,44]. As for the fourth peptide where positions 9 and 15 where substituted to alpha-aminoisobutyric acids (or alpha-methyl-lalanine (found e.g. in the antibacterial peptide alamethicin), the voltage-dependence was conserved.

In order to validate these findings on the whole channel, an electrophysiological study was undertaken on the human skeletal muscle sodium channel. The gene SCN4A was introduced into HEK293 cells and expression was monitored using the whole cell patch voltage-clamp technique. Alanine substitutions of hydrophobic residues with branched sidechains next to selected arginines and lysines were applied to engineer six mutations in the WT SCN4A cDNA (see Table 1) coding for the sodium channel isoform Nav1.4.
Electrophysiological results are summarized in Table 1. Steady-state activation was affected in domains I and II but not in domain III. While substitutions L227A, L674A and L677A shifted the activation curve to depolarizing voltages, favouring the channel closed state, for L224A there was a left shift to hyperpolarizing voltages that enhances the channel open state (Fig. 5).

In domain III, whole cell inward current at \(-10\) mV showed that L1131A channels exhibited slower inactivation kinetics whereas L1137A mutant channels had similar inactivation kinetics to that of WT channels. At the same voltage, the time constant for inactivation of the L1131A mutant is about 3-4 times slower than that of WT channels. Steady-state inactivation was shifted toward hyperpolarizing potentials for both mutations and this was accompanied by a significant reduction of the slope factor only for the L1131A (Table 1).

It should be noted that except for the L1131P mutation (outside the scope of this study), for which a reduction of the sodium current was observed [45], the amplitude of the peak inward current remained unaffected by these mutations [46].

The structure of crystallized Kv1.2 [8], a mammalian potassium voltage-dependent channel of the Shaker family, provided the template for homology modelling of domains I, II and III voltage sensors of Nav1.4 sodium channel. The positions of the S1 and S3 \(\alpha\)-helices could be determined and the resolution of a number of S4 sidechains in the Kv1.2 structure allowed the determination of the register of this \(\alpha\)-helix. Chains of residues in the S1, S2 and S3 helices are conserved in Shaker channels and participate in \(\textit{in vivo}\) electrostatic interactions [47,48]. In the model, the sidechain of L1131 is located at the interface between the S3 and S4 helices. Its mutation would therefore be expected to have not such an effect as L1131A does on the stability of this domain.

This work was focused only on S4 segments of domains I, II and III since S4 of domain IV is more involved in inactivation and its coupling to activation has already been extensively studied in the past (e.g. see [49]).

<table>
<thead>
<tr>
<th>Domain</th>
<th>Clone</th>
<th>Activation midpoint (V_{1/2}) (mV)</th>
<th>Activation slope</th>
<th>Fast Inactivation midpoint (V_{1/2}) (mV)</th>
<th>Fast Inactivation slope</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Activation slope</td>
<td>Fast Inactivation midpoint (V_{1/2}) (mV)</td>
<td>Fast Inactivation slope</td>
</tr>
<tr>
<td>D I</td>
<td>WT</td>
<td>(-17.7 \pm 0.4) (47)</td>
<td>3.8 \pm 0.5 (28)</td>
<td>(-62.1 \pm 0.7) (43)</td>
<td>(-3.9 \pm 0.7) (26)</td>
</tr>
<tr>
<td>D II</td>
<td>L224A</td>
<td>(-24.5 \pm 1.2) (13)</td>
<td>4.1 \pm 0.6 (13)*</td>
<td>(-63.1 \pm 1.2) (10)</td>
<td>-4.5 \pm 0.1 (10)</td>
</tr>
<tr>
<td>D III</td>
<td>L674A</td>
<td>(-11.3 \pm 0.8) (8)</td>
<td>2.9 \pm 0.7 (7)*</td>
<td>(-62.2 \pm 1.1) (13)*</td>
<td>-3.4 \pm 0.2 (12)</td>
</tr>
<tr>
<td>D III</td>
<td>L677A</td>
<td>(-10.7 \pm 0.7) (13)</td>
<td>3.5 \pm 0.5 (13)*</td>
<td>(-58.0 \pm 0.7) (13)</td>
<td>-3.8 \pm 0.1 (11)*</td>
</tr>
<tr>
<td>D III</td>
<td>L1131A</td>
<td>(-6.2 \pm 1.3) (9)</td>
<td>3.8 \pm 0.4 (8)*</td>
<td>(-61.6 \pm 1.5) (10)*</td>
<td>-3.9 \pm 0.2 (8)*</td>
</tr>
<tr>
<td>D III</td>
<td>L1137A</td>
<td>(-19.5 \pm 0.9) (12)*</td>
<td>3.5 \pm 0.5 (12)*</td>
<td>(-70.8 \pm 1.9) (20)</td>
<td>-2.1 \pm 0.2 (13)</td>
</tr>
<tr>
<td>D III</td>
<td>L1137A</td>
<td>(-18.9 \pm 1.5) (8)*</td>
<td>3.2 \pm 0.3 (8)</td>
<td>(-67.6 \pm 1.5) (9)</td>
<td>-3.8 \pm 0.1 (9)*</td>
</tr>
</tbody>
</table>

Values are mean \(\pm\) SEM and Student’s t-test or ANOVA was used to test for statistical difference between the groups. A value of \(P\) smaller than 0.05 was considered statistically significant. Values not considered significant are marked with asterisks. (from Bendahhou et al. [46]).
4. Summary and discussion

The common thread among the apparently different aspects reviewed here remains an interdisciplinary approach of this physiologically important ion channel and the results are compared with similar studies from the literature. Electric eel was used throughout these studies since it is a natural preparation rich in VGSCs which are homologous to other bacterial and mammalian VGSCs. Our initial aim was to crystallize eel VGSCs but this proved impossible as other groups also found. In addition, NMR does not appear suitable for large membrane proteins. This is why we had to rely on Circular Dichroism to assess the channel’s secondary structure.

The effect of deglycosylation was firstly investigated stressing the role of negatively charged carbohydrate moieties to increase the density of sodium ions near the outer mouth of the channel and deglycosylation was also found to alter the gating properties of rNav1.3: the steady-state activation was positively shifted by about 10 mV whereas inactivation was slightly negatively shifted. Then, deeper in the channel, drug binding puts forward interactions with specific residues in the pore lining. It is worth noting that new anticonvulsants targeting VGSC such as saponins from Ficus platyphylla and Lacosamide have been recently studied (respectively [50,51]). For a general review of therapeutic approaches to ion channel diseases, see [52]. It might be worthwhile to reinvestigate this
question of drug binding with deglycosylated sodium channels to unveil the effects on channel conductance substates. The role of hydrophobic residues in voltage sensors demonstrated activation and inactivation changes, sometimes comparable to the well-known effects of mutations of positively charged residues.

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References


