A complicated complex: ion channels, voltage sensing, cell membranes and peptide inhibitors

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Abstract

Voltage-gated ion channels (VGICs) are specialised ion channels that have a voltage dependent mode of action, where ion conduction, or gating, is controlled by a voltage-sensing mechanism. VGICs are critical for electrical signalling and are therefore important pharmacological targets. Among these, voltage-gated sodium channels (Na_Vs) have attracted particular attention as potential analgesic targets. Na_Vs, however, comprise several structurally similar subtypes with unique localisations and distinct functions, ranging from amplification of action potentials in nociception (e.g. $Na_V1.7$) to controlling electrical signalling in cardiac function ($Na_V1.5$). Understanding the structural basis of Na_V function is therefore of great significance, both to our knowledge of electrical signalling and in development of subtype and state selective drugs. An important tool in this pursuit has been the use of peptides from animal venoms as selective Na_V modulators. In this review, we look at peptides, particularly from spider venoms, that inhibit Na_Vs by binding to the voltage sensing domain (VSD) of this channel, known as gating modifier toxins (GMT). In the first part of the review, we look at the structural determinants of voltage sensing in VGICs, the gating cycle and the conformational changes that accompany VSD movement. Next, the modulation of the analgesic target $Na_V1.7$ by GMTs is reviewed to develop bioinformatic tools that, based on sequence information alone, can identify toxins that are likely to inhibit this channel. The same approach is also used to define VSD sequences, other than that from $Na_V1.7$, which are likely to be sensitive to this class of toxins. The final section of the review focuses on the important role of the cellular membrane in channel modulation and also how the lipid composition affects measurements of peptide-channel interactions both in binding kinetics measurements in solution and in cell-based functional assays.

Keywords: Voltage-gated ion channel, venom peptide, gating modifier toxins, receptor-ligand complex, lipid binding, cell membrane.

1 Introduction

Voltage-gated ion channels (VGICs), belong to a superfamily of signalling proteins and are present in all living cells [1]. Isoforms of these VGICs are also known to be expressed in metastatic cells from a number of cancers [2]. These channels principally function to mediate conduction of ions across the membrane, and are involved in a wide range of physiological functions. The VGIC superfamily is encoded by more than 143 genes in the human genome, making them the third largest family of cell signaling molecules after GPCRs and kinases [1, 3]. The primary role of these ion channels is in communication of electrical signals in the nervous system, where they are responsible for the generation and transduction of action potentials. The dysfunction of these VGICs can lead to a multitude of hereditary and congenital channelopathies including epilepsy, chronic pain, cardiac disorders, autism spectrum disorder, Schizophrenia and Alzheimer's disease [4]. Unsurprisingly, these ion channels are also among the most common drug targets. Despite their ubiquitous involvement in a multitude of disease states, currently only around 30 ion channel drugs are in the market, targeting less than 10 of the known ion channel subunits [4]. This means that although ion channels are one of the most common drug targets, their potential is far from fulfilled.

Perhaps the most intensely pursued VGIC for drug development is $Na_V1.7$, an isoform of the voltage-gated sodium channel family [5], which compelling genetic evidence has identified as a promising target for the development of analgesic drugs. The structure of Na_Vs comprises an ion-conducting pore surrounded by four voltage-sensing domains (VSDs). The VSDs are responsible for sensing the polarization of the membrane and will undergo a structural change when the membrane polarization changes. This conformational change is coupled to the opening and closing of the channel pore. There is high sequence conservation in the ion-conducting pore of different subtypes of Na_Vs, while the VSDs of these channels display a higher sequence divergence [6]. There has, therefore, been a particular focus on identifying ligands that can modulate channel function through the allosteric, VSD mediated, mechanism of channel gating [7]. VSD ligands can indirectly serve to stabilize different states of the channel without binding to the pore. A number of such allosteric, gating modifier, ligands have been identified with a few in various stages of clinical trials [8, 9].

Although $Na_V1.7$ inhibitors have been pursued intensely for the past decade, there are still significant gaps in our knowledge regarding the mechanism of voltage sensing [10]. This knowledge gap is in large part due to the lack of structural data of the channel in its resting state, which only exists in the presence of a membrane potential or a suitable ligand. A membrane potential is difficult to establish in the biophysical experiments used to study these channels in solution. Selective and potent ligands, such as animal venom peptides (toxins), thus represent not just potential drug leads but also important biophysical tools for the study of VGICs. In this review, we focus on gating modifiers that stabilize the elusive resting state of the VSD [11], and use a combination of bioinformatic and kinetic analyses to summarize the wealth of literature ranging from biophysical to cell-based investigations, with a particular focus on spider toxins that modulate the gating behavior of $Na_V1.7$. The review is organized by considering the three components in the channel:ligand complex: (*i*) the first section introduces VGICs as a structural class and identifies those that are likely to be involved in voltage gating. This part also covers the channel architecture and the gating cycle; (*ii*) in the second part we look at the structure-activity studies that have been done using venom peptides that stabilize $Na_V1.7$ in the resting state, look at the available mutagenesis data from both the toxin and the channel and use bioinformatics to identify predictors of binding; (*iii*) in the final section we look at how the membrane itself can affect the binding kinetics of channel inhibition by gating modifiers, and we also look at the membrane composition of different cell types and how this may influence binding measurements. We hope to provide an overview of what has

been gleaned from a wealth of structural and functional studies and point to some of the complicating factors that have made capturing the structure of the resting state of the channel difficult.

1.1. VGIC architecture

VGICs are integral membrane proteins with diverse structures. The general architecture of VGICs, however, comprises a pore forming α-subunit comprising 24 transmembrane helices. The gating and kinetics of the α-subunit may be controlled by auxiliary proteins (e.g. β-subunits in Na_Vs) [12]. VGICs can either exist as tetramers (e.g. voltage-gated potassium channels (K_Vs) or prokaryotic Na_Vs) or as monomers with four homologous domains (e.g. eukaryotic Na_Vs and voltage-gated calcium channels (Ca_Vs)). Tetrameric channels can either be homomeric or heteromeric. Each domain contains six transmembrane (TM) segments (S1–S6) joined by intra- or extracellular loops. The four domains fold together in a circular orientation such that domains I and IV are brought into close proximity. The S5-S6 segments from each of the four domains come together to form a pore domain (PD). The pore domain comprises a wide outer vestibule on the extracellular side, a selectivity filter (upper gate), a large water filled central cavity and an intracellular activation gate (lower gate). The conformational changes in the upper and lower gates control ion conduction. The S1-S4 segment of each domain forms a VSD where the positively-charged S4 segments serve as voltage sensors that initiate voltage-dependent activation by moving outward under the influence of changes in membrane potential [13]. This movement controls the opening of the channel. The VSD of each domain interfaces the PD of the neighbouring domain (Fig. 1).The channels may have additional extracellular or intracellular domains depending on their specific functions [12].

[Figure 1 near here]

VGIC are classified in families by the type of ion they conduct and each family typically contains multiple isoforms or subtypes, each with different signalling properties and tissue distribution. In humans, voltage gated sodium channels (Na_Vs) are classified into nine different subtypes, denoted $Na_V1.1$ to 1.9 [14]. The α-subunit of mammalian Na_Vs comprise four internally homologous but non-identical domains (denoted domains I to IV) connected by intracellular linkers (Fig. 1).

The pore domain (PD) and the VSD only share a small interface and it has been shown that the structure of the VSD (S1-S4) is unperturbed when studied in isolation [14]. This modular architecture is consistent with the observation that VSDs occur naturally as domains in enzymes and can exist without auxiliary domains, as is the case for the proton channel H_V1 and voltage sensing phosphatase [15-17]. Conversely ion-channels exist that only contain the PD without the VSD [18]. Finally, it has been shown that it is possible to transfer segments of the VSD from one type of ion-channel to another without loss of ion-selectivity or activity [19]. Thus, the VSD of VGICs can be regarded as independent structural domains. Much of the structural characterisation of ion channels have to date been from X-ray structures of symmetrical homo-tetrameric channels from prokaryotes. However, recent advances in cryo-EM have resulted in the emergence of structures of more complex monomeric channels from eukaryotes [20].

The VGICs can be either inhibited by blocking the ion-conducting pore or through modulation of the VSDs. Here, we are particularly interested in peptides that bind to the less well-conserved VSD. In the following section, we will discuss VGICs that contain VSDs that are likely to respond to membrane voltage changes and therefore likely pharmacological targets.

1.2 Not all voltage-gated ion channels are voltage gated

VGICs as defined by IUPHAR constitute a diverse structural family of proteins (Table 1) [21]. The majority of these channels have some voltage sensitivity, but some have VSDs that respond weakly or not at all to physiological changes in membrane potential. As can be seen in Table 1 some VGICs do not have VSDs and some that do are ligand gated and in the absence of these ligands may not be voltage sensitive (e.g. TRP channels). Voltage gated sodium (Na_VS), potassium (K_VS), calcium (Ca_VS – including the related CatSper/TP channels) and proton (H_VS) channels contain VSDs that are voltage sensitive in the physiological range and in the absence of ligands. This voltage sensitivity is mediated by several positive charges in the VSD, typically in the form of 3 to 6 arginine or lysine residues in the C-terminal transmembrane helix of this domain (referred to as gating charges). Therefore, the classification in Table 1 appears to be driven by sequence homology rather than a rational basis of voltage gating.

[Table 1 near here]

The focus of this review is the modulation of VGIC function by peptides that modify the voltage gating behaviour of the channel. In this regard, we are only concerned with VGICs that have a VSD that can influence the gating behaviour of the channel. Since many of the channels classified as VGICs do not have voltage sensing VSDs, it is more informative for us to define a subset of VGICs as "voltage-sensing proteins", defined as proteins containing a conserved voltage sensor. This classification has previously been defined by Palovcak *et al* and applying the same sequence alignment criteria (HMMER profile) we find a total of 80 known human genes that are identified as putative voltage-sensing proteins by this tool, including non-ion-channel proteins (Table 2) [22]. These proteins are all predicted to be susceptible to allosteric regulation through their VSDs.

[Table 2 near here]

In Table 2, we observe that only a subset of what are considered VGICs are actually likely to be voltage sensitive, and there are a number of other proteins not considered VGICs that better fit into this group. For example, transient receptor potential (Trp) channels have a four-helix domain that topologically resembles a VSD (Table 1), but this domain is not identified as a VSD based on the conserved sequence characteristics of a voltage-sensing VSD (based on the HMMER profile). We also find that even those identified as having the conserved voltage sensing VSD sequence [22] do not all contain gating charges (e.g. $K_{Ca}1.1$) and are therefore likely to be either weakly voltage sensing or not voltage sensing at all. From Table 2, we would predict that all sequences containing 4 or more gating charges are likely to be susceptible to allosteric gating by VSD binding ligands, but those containing 3 or less gating charges may or may not be susceptible to gating modifier ligands. We also note that the 80 sequences in Table

2 are from human proteins that have been curated in UniProt. If the entire UniProt database is probed using the VSD search tool, we find an additional 37 sequences in the human genome (not shown) that are predicted to contain a VSD, but are yet to be characterised.

1.3 The VSD in a polarised membrane

The electrical potential across the membrane is generated by differences in the concentration of different ions on either side of the membrane. The extracellular side has a net positive charge compared to the intracellular side (or conversely, the intracellular side has a net negative charge). This inequality is due to pumps that actively transport ions across the cell membrane against their electrochemical gradient, resulting in an electrical potential difference between the two sides of the membrane. The hydrocarbon tails of the lipids are weakly polarisable and effectively act as insulation [7]. Outside the membrane the field can be considered constant and the movement of charged particles here, will not be affected by the potential difference. Proteins that span the membrane, however, are polarisable and these will be affected by the difference in electrical potential between the two sides. Helical proteins additionally have a natural dipole that runs from the positive N-terminus to the negative C-terminus, which is due to the polar carbonyl groups aligning perpendicular to the plane of the membrane. Thus, it is energetically more favourable for a helix that spans a polarised membrane to be oriented such that its N-terminus is on the intracellular side with the C-terminus on the extracellular side (compared to the reverse) [23]. In a VSD the S1 and S3 helices are in a favourable alignment with the C=O groups pointing towards the more positive extracellular environment, while the S2 and S4 helices are in an unfavourable alignment with the helix dipole pointing in the opposite direction to the voltage potential across the membrane. This would suggest that there is a force that would tilt the S2 and S4 helices towards the plane of the membrane (as they would prefer to 'flip'), while the S1 and S3 helices would favour to be perpendicular to the plane of the membrane [24]. In the absence of a membrane potential there would be no particular force that would interact with the helix dipole, and we propose that this may be sufficient to make the protein more dynamic (than it is in the presence of a membrane potential). This proposed plasticity of the depolarised state is consistent with the structural heterogeneity of VSDs observed in structural studies of VGICs.

The sidechains themselves are also polarisable and their importance is highlighted by the extensive bioinformatic analysis by Palovcak *et al* [22], which shows that the following regions of the VSD are highly conserved:

i. *Basic residues separated by 3 amino acids in S4.*
ii. *One acidic residue in S2, one acidic residue or asparagine in S2 and one acidic residue in S3.*
iii. *An aromatic residue between the two acidic residues in S2 and facing a hydrophobic residue in S1.*

In the polarised membrane, many basic residues that line the S4 helix (point *i* above) will be attracted to the intracellular anionic side of the membrane, thus exerting a further force (in addition to the helix dipole) to move inward towards the more negative side of the membrane. Similar to the argument above regarding the helix dipole, this force would once again be removed when the membrane becomes depolarised and the S4 helix would shift away from the intracellular side [25]. The movement of the S4 charges across a hydrophobic membrane would be energetically very costly, and the presence of the counter charges (see point *ii* above) provides a low energy pathway for this movement to occur [13]. The movements of the S4 helix have previously been modelled, where it was shown that arginine residues in the S4

segment (including those in (*i*) above) change their interactions with residues in the intracellular negative charge cluster (INC) to those in the extracellular negative charge cluster (ENC) – including those residues in (*ii*) above. It was further shown that a focused electric field between the extracellular and intracellular side of the membrane was generated by the hydrophobic constriction site (HCS) that plays a crucial role in the translocation of the gating charges from the INC to ENC resulting in outward movement of the S4 transmembrane segment. The modelling further showed that the translocation of the S4 segment generates a concerted conformational change in the pore domain that allows the intracellular activation gate to open and conduct ions in the open state [26].

Combining the effect of the helix dipole with the S4 basic charges, it is likely that the S4 helix undergoes both a tilt and a shift in response to the change in membrane polarisation. This is consistent with both the modelling discussed above and EPR studies of the KvAP channel in solution where the membrane potential was artificially mimicked through protein-lipid interactions [27]. It should be stressed that all the existing high-resolution structural data is in the absence of a membrane potential. Thus, models describing the structure of the VSD in the presence of a membrane potential remain to be confirmed.

Finally, we note that the helices have hydrophobic residues facing the membrane with the lining of the VSD containing largely polar amino acids. The presence of these polar residues suggests that the VSD interior is water accessible with the conserved hydrophobic residues, noted above in point (*iii*), near the centre of the VSD providing a hydrophobic division between the extra- and intra-cellular solution [27]. There are also conserved regions in the N- and C-termini, but these are likely important for anchoring the protein in the membrane.

[Figure 2 near here]

1.4 The gating cycle

In the above section the anatomy of the channel is discussed as well as the mechanics of the forces that influence movement of the VSD, but how does this relate to the function of the channel and how do ligands influence this? For many proteins/enzymes the structure of the protein in solution represents a state that does not change much in response to ligand binding, and often a ligand can be "docked" onto/into the protein *in silico* or indeed the ligand can be experimentally "soaked" into a protein crystal, in both cases pointing to minor conformational changes required to accommodate the ligand [28, 29]. This type of behaviour has been of great utility in drug discovery and development. Transmembrane proteins, that often act to transport signals or cargo across the membrane, are notorious for not conforming to this pharmaceutically useful behaviour. They can (and very often will) undergo significant conformational changes in response to a number of factors such as auxiliary proteins, ligands, membrane composition and membrane polarisation, to name a few. VGICs are an excellent incarnation of the more complex and dynamic scenario, as the very existence of these signal transduction proteins relies on relatively large conformational changes that lead to opening and closing of the ion conducting pore.

It may at this stage seem like we can categorise the structure of the VGICs as either open or closed. The *closed* state is preferred in a polarised membrane and is also known as the "resting" state. In this state, the S4 segment of the VSD would be drawn to the intracellular side by the gating charges and this state is therefore sometimes also referred to as the "down" state. This conformation prevents the channel gate from opening. When the membrane is depolarised, the channel will transition to an *open* state. There is no longer any force acting on the gating

charges of the VSD, and it can transition from the down state to a more relaxed "up" state, allowing the channel gate to open and ions to pass. In reality, there are more than these two states, as channel opening needs to be fine-tuned such that each channel is only open for a distinct period of time, and such that prolonged depolarisation does not lead to persistent currents [30]. To control this, the channel transitions from the open state to an "inactivated" state – a process referred to as fast inactivation. In this state, the channel is occluded by an alternative mechanism. In Na_VS, this mechanism is controlled by movement of the VSD of domain IV which moves slower than the VSDs of the other domains in response to depolarisation. This movements results in occlusion of the pore by an inactivation particle (or IFM particle) in the intracellular domain III-IV loop. In the inactivated state the channel is thus in an open, but non-conducting state, and the VSDs are all in the "up" state since the membrane is depolarised. Once the membrane is repolarised, the channel will transition through a deactivated state where the channel gate is closed and the channel is inactivated, subsequently returning back to the resting state [13]. In the simplest terms the gating cycle can be considered as transitions from the closed state to the open state, and then the inactivated state, through the deactivated state before finally returning back to the closed state (Figure 3).

[Figure 3 near here]

Even this very simple model has multiple conformational states with complex and dynamic equilibria. In reality, the problem is actually more complicated; the above model for the VGIC must be expanded with additional conformational states and equilibrium constants to account for: *i*) fast *vs* slow inactivation, *ii*) auxiliary subunits and *iii*) membrane composition. The equilibria are further sensitive to pH, temperature and the concentration of various ions in the solution. For the channels that have four distinct VSDs (see tables 1 & 2 – note that this includes heteromeric K_VS) the sensitivity of each VSD to the above parameters must also be taken into account. Although heroic attempts have been made to deconvolute all possible equilibria in mathematical models, the number of parameters very quickly exceeds what can be controlled under standard experimental conditions, and it is often easier to simplify the model to the three-state model (closed-open-inactivated), ignoring all augmenting variables [31, 32]. In the context of this review we will discuss how venom peptides modulate channel function and this simpler model is nearly always sufficient. In Figure 3, we note particularly that those peptides that inhibit the channel (and are therefore of particular pharmacological interest) can do this by either stabilising the resting or inactivated states of the channel. This involves either trapping the VSD responsible for channel opening in the resting, down, state, or trapping the VSD responsible for return of the channel to the resting, in the inactivated, up, state – see also section 2.1. In $Na_V1.7$, this corresponds to stabilising the VSD of domain II (VSD-II) in the down state or stabilising the VSD-IV in the up state. Structural characterisation of the latter has been achieved in channel chimeras [33] but the former remains a state difficult to access by high resolution structural studies, and poses a significant hurdle in both understanding the structural basis of voltage sensing and for rational drug design. Although the VSDs of domains I and III are also involved in the gating cycle, we will not discuss these further as they are currently not known to be the sites of any channel inhibitors.

2. Structure activity studies of VSD-peptide interactions

2.1 Modulation of voltage sensing by venom peptides

In the previous section, we described the VSD of VGICs and in particular noted the difficulties in accessing structural information regarding channel inhibition by stabilising the down state of the VSD. In Na_Vs this would involve binding to the VSD-DII of the ion channel in the elusive resting/polarised/down state of the channel. Instead, much of what is known about this route of channel inhibition has been gleaned by exploring the mechanism of channel inhibition by venom peptides [10, 34-37]. In this section, we will look at this fascinating class of compounds and in particular focus on structure-activity studies revealing the details of peptide-channel interaction involving the resting state of the VSD-DII of Na_Vs.

A recent survey of all reported venom peptide revealed that there are ~1,000 ion-channel modulators reported to date [10]. Of these, 407 are Na_V channel modulators, 278 are K_V modulators and 98 are Ca_V channel modulators. Scorpion venoms appear to be a particularly rich source of VGIC modulators with ~350 reported peptides, followed by spiders with ~200 peptides. This analysis revealed that Na_Vs have been particularly well represented in studies of venom peptides. Many peptides from both scorpions and spiders have been found to modulate these ion channels through interaction with the VSDs. This is in contrast to cone snail venom peptides that inhibit VGICs by binding to the pore of the channel [38]. Pharmacologically, pore blockers would act in a similar manner to existing pharmaceuticals such as local anaesthetics (although their binding sites are not the same). Peptides that modulate the channel through binding to the VSD, therefore present a novel mechanism of ion-channel modulation. It is in part due to this unique mode-of-action that venom peptides have attracted great interest from academia and the pharmaceutical industry alike as a source of VGIC modulators [34, 39].

Nowhere is this interest more evident than the, now more than, decade long search for a selective peptide inhibitor of $Na_V1.7$. $Na_V1.7$ has attracted attention as a drug target due to a number of reports in the mid 2000s conclusively linking loss-of-function mutations in this channel to congenital insensitivity to pain (CIP) in humans [40]. This provides compelling evidence that inhibition of $Na_V1.7$ would lead to analgesia. The 1.7 subtype is, however, only one of nine Na_V subtypes and inhibition of many of the other subtypes would lead to unacceptable adverse effects including ataxia and cardiac arrhythmia/arrest, creating a narrow therapeutic window for poorly selective ligands [38]. Peptides that bind to the VSD of $Na_V1.7$ are considered particularly attractive since this region of the channel is less conserved than the pore region, and it is hoped that higher selectivity can be achieved by so called gating modifier toxins (GMTs) that bind to the VSD of VGICs [38, 41].

From the simple model in Figure 3 we see that we can achieve channel inhibition by either stabilising the closed or inactivated state of the VSD. Binding to the inactivated state requires that the GMT binds to the channel after the channel has undergone at least one gating cycle, whereas GMTs that bind to the closed state are more attractive since they bind to the channel in its resting state and do not require the channel to have undergone a gating cycle. One of the first and most promising peptides reported that binds to the VSD of $Na_V1.7$ was ProTx-II isolated from the venom of the tarantula *Thrixopelma pruriens* [42, 43]. Almost in parallel, another spider venom peptide, HwTx-IV, was described to also act as a GMT [44, 45].

Both toxins are disulfide rich peptides that fold into the well characterised inhibitor cystine knot (ICK) motif, although they have low sequence identity beyond their cystine framework. Nevertheless, both of these peptides were initially found to bind to the VSD-DII of $Na_V1.7$. Later, however, it was shown that their binding site on VSD-DII of $Na_V1.7$ only partially overlapped, suggesting that they have slightly different channel recognition motifs [46]. Furthermore, it was found that ProTx-II in addition to binding to the VSD-DII also binds to the VSD-DIV of $Na_V1.7$ [46].

Using the simple model in Figure 3, binding to VSD-DII in Na_Vs affects the transition from the closed state to the open state, effectively trapping the channel in the resting state. Binding to VSD-DIV has a more complex pharmacology, resulting in two contrasting outcomes. In one case binding to DIV can affect the transition from the open to the inactivated state, which would lead to unwanted persistent currents and may indeed be the desired pharmacological outcome from the venomous creature's point of view [47]. On the other hand, binding to DIV may also impede the transition from the inactivated state to the resting state, thus not allowing the channel to respond to subsequent depolarisation. The second scenario also leads to channel inhibition, and has been shown to be the mechanism by which recently developed sulfonamide ligands inhibit the channel [33].

Interestingly, these two distinct pharmacological outcomes suggest that the VSD has a different conformation in the open and the inactivated state (i.e. two distinct 'up' states), both in the absence of membrane polarisation. Indeed, recent work in characterising the binding of another spider venom peptide, VSTx1, to the VSD of the archeal K_V channel KvAP have provided evidence that the VSD is dynamic and sensitive to the binding of the toxin [48-50].

2.2 HwTx-IV sensitive VSDs

Structural details of the down state of Na_Vs remain speculative and even more so in the presence of a peptide ligand. There is, however, detailed mutagenesis work which has revealed the amino acids in these channels that make them sensitive to HwTx-IV [37]. In this section, we will look at these structure-activity-relationship (SAR) studies and use the information from these in combination with sequence alignments to predict more broadly what VGIC VSDs are likely to be sensitive to toxins such as HwTx-IV. This information will inform what channels will be important to counter-screen against when developing selective VSD-DII ligands that have a similar binding mode to HwTx-IV.

The comprehensive mutagenesis work by the Cummins group showed that five residues of $Na_V1.7$-VSD-II were particularly important for peptide biding – E753, E811, L814, D816 and E818 (EELDE). The first of these is in the S1-S2 region of the channel, while the other four are in the S3-S4 region, placing the toxin inside the cavity formed between the four helices of the VSD (note that none of these correspond to the conserved residues shown in Figure 2). This mode of binding is also consistent with recent solution state and mutagenesis studies of other related systems [36, 48]. Given that we have in Table 2 generated a list of all VSDs aligned according to their shared structural homology, and we know what residues are determinants for HwTx-IV sensitivity, it is in principle now possible to query what VSDs are likely to bind the toxin. If we classify all the VSDs in Table 2 based on how many of the EELDE motif they have (allowing D to E mutations and L to A/I/V mutations), and we apply a strict filter requiring the final acidic residue (as shown by previous studies to be required) we arrive at the data summarised in Table 3. We can then rank likely HwTx-IV sensitive VSDs based on how many of the required residues they contain.

[Table 3 near here]

The data in Table 3 is notable because the subtype selectivity of HwTx-IV for Na_Vs is well characterised and it is known to bind 1.7, 1.6, 1.1, 1.2 and 1.3 channels only [51], as predicted from the above table. The activity of the toxin on $Ca_V1.3$ has not been described in the literature

to the best knowledge of the authors, but our analysis suggests that the peptide will bind strongly to this channel as well as several of the Ca_V3 subtypes.

2.3 $Na_V1.7$-DII binding peptides

The analysis done in the above section relies on detailed mutagenesis of the VSD with corresponding activity data as well as a good alignment template. HwTx-IV is itself extremely well characterised in terms of mutagenesis studies mapping the residues required for the activity of the toxin against $Na_V1.7$. Similarly, alignments of the ICK fold have been described in detail and alignment templates have been generated [34, 35, 52]. Thus, it is possible to apply a similar analysis as above to identify toxins that are likely to act as inhibitors of $Na_V1.7$.

Residues K31/W29 are absolutely required for activity of HwTx-IV. S24 is required to position K31/W29 correctly, and a hydrophobic residue in either position 7 or 8 is important for membrane association and may be important for VSD binding as well [53]. This provides us with $X_1X_2SWCKX_3$ motif that is essential for the activity of the peptide on $Na_V1.7$. The K and W residues are known to be essential for the activity of the peptide while the other residues all have additive effects on the activity. Allowing for any hydrophobic residue (I, L, V, M, F, W or Y) in place of X and removing those that lack the KCW motif results in a list of potential $Na_V1.7$ inhibitors (Table 4).

[Table 4 near here.]

In Table 4 we find, as expected, that HwTx-IV (Hs2a) has all the critical residues (7), but we also find that Hhn2b has 5 of the 7 required residues. Hhn2b is known to be inactive on $Na_V1.7$ and indeed the two missing residues (X_2 and S) were shown to each contribute independently to the lack of activity. If either of the two residues (G and N) is mutated into the corresponding required residues, the peptide regains activity (albeit with a lower potency), and if both are mutated to the corresponding required residues, the protein has a similar potency to HwTx-IV [53]. Thus, although our analysis would suggest that all of the toxins with 7 recognition residues should inhibit $Na_V1.7$, and those with 6 residues should all produce activity but perhaps with some reduced potency, we note that those with 5 may or may not inhibit $Na_V1.7$ depending on how additive the effect of the missing residues are.

Nevertheless, the training of a sequence alignment tool and the subsequent sequence analysis complemented with information from detailed mutagenesis yields a remarkable result in that most of the peptides in Table 4 are known inhibitors of Na_Vs. This would allow future transcriptomic and proteomic data be processed using these tools to identify potential $Na_V1.7$ inhibitors that act with a similar mechanism of action as HwTx-IV. Interestingly HwTx-I (Hs1a) and Gr2a were initially identified as calcium channel inhibitors and later also found to inhibit sodium channels. If we combine the information from Tables 3 and 4 we may conclude that many of the peptides in Table 4 are likely to bind to the VSDs of the top hits in Table 3, which are primarily Na_Vs and Ca_Vs.

2.4 In silico screening of venoms

The above analysis to identify peptide sensitive VSDs and VSD binding peptides raises the question of whether future screening efforts can be conducted *in silico*. It should be noted that the bioinformatic analysis performed here is only possible due to the availability of a wealth of

functional data from detailed mutagenesis data. Thus, the analysis is only applicable to known and well characterised families of ligands and receptors.

The argument can be made that such *in silico* analysis will simply identify variants of known inhibitors. The counter argument is that it is exceptionally rare to find a completely new class of peptides that target a well-established receptor via a novel mechanism. For example, despite extensive screening of venoms for $Na_V1.7$ inhibitors, all reported inhibitors fall within either the HwTx-IV family or the ProTx-II families (the reported centipede toxin is excluded as its Na_V inhibition cannot be confirmed independently [54]). This includes a recent screen of 205 venoms where all the characterised and reported hits fell within the sequence space that would have been identified by the analysis in section 2.3 [39]. It would, therefore, appear that for new targets, there would be significant value in initial screening of venoms to identify consistent hits. Once these are identified, however, the effort should be focused on establishing detailed SAR data through careful mutagenesis.

The benefit of performing *in silico* screening of venoms for a known and well characterised ligand-target pair is that the wealth of sequence data provides a database of sequence variants that have been optimised by evolution and are likely to be stable and soluble. In contrast, simple alanine or arginine scanning, can often lead to insoluble or misfolded peptides. The natural sequence variation will also point to regions that are tolerant to mutations, insertions and deletions – information that is otherwise very difficult to acquire. In the case of finding a $Na_V1.7$ inhibitor that has the desired analgesic effect, the inhibitor is required to be VGIC and subtype selective. The natural sequence variants identified through analysis of transcriptomic data provides an ideal starting point for addressing this. Thus, although *in silico* screening will not identify new classes of molecules, it provides an excellent tool in the optimisation process of known ligand classes.

An example of the sequence diversity apparent from our analysis is that the first of the 7 residues in Table 4 is uniquely a proline in P0CH54. This may have a significant effect on the structure of the first loop in the peptide and would be an interesting mutation to explore. Similarly, the sequence alignment of the sequences in Table 4 (not shown) reveals a highly conserved proline residue which is placed two residues after the second cysteine, however, in both P0CH54 and B1P1H2 this residue is a tyrosine. These are only a few observation and further natural sequence variations are apparent in different positions, that would otherwise be difficult to arrive at by existing mutagenesis approaches.

3. Binding kinetics in the presence of a lipid bilayer

3.1 Binding kinetics of spider toxin gating modifiers

In the previous sections, we have discussed in some detail the SAR studies that underpin our understanding of channel inhibition by GMTs. However, many of the determinants of toxin activity may not just purely involve protein-protein interactions. This is evident in studies showing that toxin activity cannot be fully explained by channel:peptide binding kinetics in the absence of a lipid bilayer [55]. The lipid bilayer, thus, plays a significant role in toxin binding and many of the GMTs described to date have also been shown to bind to lipid bilayers [53, 55, 56]. Further, the lipid bilayer composition has been shown to modulate the gating mechanism of the channel [57, 58]. In this section, we will first briefly review the literature on lipid binding of GMTs and in the subsequent two sections discuss the impact of the lipid bilayer on the binding kinetics of GMTs (3.2) and then review the lipid composition of cells that are both the target of GMTs and those commonly used in functional assays (3.3).

One of the earliest reports on the effects of the lipid bilayer on binding kinetics of GMTs was from the MacKinnon group, where they describe the interaction of a GMT (VSTx1) with a voltage gated potassium channel (KvAP) as well as its high affinity for lipid like micelles and bilayers [55]. Similarly, GMTs such as hanatoxin [59, 60], ProTx-II [61], HHN2B-G7W [53] and SGTx [62] have been shown to bind to lipid bilayers. These and several other studies have also consistently shown that these cationic GMTs have an increased affinity for anionic lipids [48, 55, 63, 64]. The distribution of the basic residues on the GMTs forms a basic patch surrounded by hydrophobic residues creating an amphipathic structure which has been suggested to mediates membrane association by several biophysical studies as well as molecular dynamics simulations [48, 64-66].

There is therefore a wealth of evidence, suggesting that lipid binding plays an important role in the pharmacology of GMTs. The exact mechanism, however, is still under some debate. Some of the different mechanism considered are that (*i*) the GMTs affinity for the lipid bilayer has a concentrating effect, localising the peptide near the channel, (*ii*) individual lipid molecules that are associated strongly with the channel form part of the channel-toxin complex in a "trimolecular complex", or (*iii*) the peptide partitions into the bilayer followed by lateral diffusion in the membrane followed by binding to a primarily lipid accessible binding site ("membrane-access" mechanism) [7, 55]. In the absence of high resolution structural data of a VSD-GMT complex, the contributions of mechanisms (*ii*) and (*iii*) are difficult to quantitate. In contrast, mechanism (*i*) makes no assumptions about specific intermolecular interactions and can be quantitated by classical kinetic models as described below.

3.2 'Membrane catalysis' as a kinetic model for contribution of lipid binding in channel inhibition

Lipid partitioning of ligands leading to a 'concentrating effect' is well known and models to account for this have been proposed, with the concept of 'membrane catalysis' introduced by Sargent and Schwyzer [67]. There is now growing evidence to support this phenomenon in various ligand-receptor systems [68-70]. The model has also been discussed and refined in various reviews and can be summarised in 3 steps: (*i*) accumulation of the ligand to the membrane surface by favourable electrostatic interactions, (*ii*) partial or complete entry into the membrane through hydrophobic interactions and (*iii*) ligand-receptor interactions.

Based on the membrane catalysis model, we can build a simple and practical model to draw some very general conclusions regarding GMT-VSD interactions. In this model, the local bilayer is considered as an independent entity in the three-component system (ligand, receptor and membrane). In the simplest case when not considering the effect of the membrane, the equilibrium between the GMT and the VSD follows a 1:1 non-competitive binding mode:

$$L_f + R_f \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} RL \quad (1)$$

$$K_d = \frac{k_{off}}{k_{on}} = \frac{[R]_f[L]_f}{[RL]} \quad (2)$$

where L is the ligand (i.e. the GMT), R is the receptor (i.e. the VSD), $[R]_f$ is the concentration of the free R, $[L]_f$ is the concentration of the free L, [RL] is the complex concentration and the equilibrium dissociation constant, K_d, is the ratio of the association constant, k_{on} and dissociation constant, k_{off}.

The local ligand concentration near the membrane, and thus the receptor, can be significantly increased due to (i) electrostatic and/or (ii) hydrophobic interactions. These interactions can stabilize the ligand to a membrane upon binding, lowering the ligand k_{off}. This leads to an increased or 'accumulated' population of ligands near the local bilayer and receptors (i.e. partitioning). Ligands accumulated due to (i) and (ii) can be defined as having a distinct concentration in the bilayer compartment, higher than that of the bulk aqueous volume, $[L]_f$. If we approximate processes (i) and (ii) as mechanisms of concentrating the ligand, then we can describe them as a combined partitioning equilibrium referred to as K_p. The K_p is defined by the ratio of partitioned ligand concentration (in moles per *membrane compartment volume,* $[L]_m$), over ligand concentration in the bulk solution (in moles per solution volume, $[L]_f$):

$$K_p = \frac{[L]_m}{[L]_f} \qquad (3)$$

The K_p is essentially a multiplicative factor of total ligand concentration, therefore reflecting a concentrating effect [71]. Note that the model does not implicitly make any assumptions about the location of the binding site of the channel or the GMT with respect to the bilayer, and in the GMT-VSD case there is not enough experimental evidence to make any assertions about this. Furthermore, we cannot make any assumptions regarding the role of the membrane in preconfiguring the conformation or orientation of the peptide with respect to channel binding (a slight departure from the membrane catalysis model). Finally, we have here taken a very generous view of what the *membrane compartment volume* is. It is, here, considered to extend as far as favourable electrostatic contributions can exist between any sidechains of the peptide and the outer membrane leaflet (including associated ions). Thus, a hydrophilic peptide can still accumulate near the bilayer surface through electrostatic interactions alone.

[Figure 4 near here]

Many GMTs have K_p values in the range of ~10^6 and can thus significantly affect binding kinetics. To account for the contributions of K_p to the simple model presented by equations 1 and 2, we use the membrane catalysis model to break down the binding event into two separate steady-state equilibria:

$$L_f + \ Bilayer \ \underset{\rightleftharpoons}{^{K_p}} \ L_m \ + \ R_f \ \underset{\rightleftharpoons}{^{K_d'}} \ \ RL \ + \ Bilayer \qquad (5)$$

According to this model we assume that the concentration of free ligand ($[L]_f$) will be insignificant compared to partitioned ligands ($[L]_m$) and the interaction of free ligands with the receptor can be neglected. Therefore, the K_p and K_d'contribute to an observed equilibrium dissociation constant ($K_{d(app)}$):

$$K_{d(app)} = \frac{K_d'}{K_p} \qquad (6)$$

$K_{(d)app}$ is a useful representation of binding kinetics for a lipophilic ligand that accounts for separate K_p and K_d equilibria. Each of these separate equilibria can in theory be determined individually by *in vitro* experiments in solution. Assuming that GMT binding to the VSD leads to complete channel inhibition as measured by ion conduction, $K_{d(app)}$ can be correlated to IC_{50} values from electrophysiology experiments:

$$[IC_{50}] \propto K_{(d)app} \quad (7)$$

We stress that this correlation provides only a rough estimate and that these values will further be modulated depending on the population of different states of the channel the affinity of the ligand to these states and their effect on the observed ion conduction. Furthermore, we note that VGICs have four VSDs and in the above we assume that binding to one VSD is adequate for total modulation of a channel, and suitable modifications may be necessary to account for stoichiometry when the above is not true. The above models have been applied to the binding of VSTx1 to KvAP [55]. Electrophysiology experiments performed using KvAP in artificial bilayers of 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) and 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (POPG) composition yielded an IC_{50} of ~10 nM [55, 72, 73]. Further, K_p values for the partitioning of VSTx1 in bilayers of 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and POPG in liposomes have been determined with values around 10^4 reported [48, 55, 63, 65].

Assuming that $K_{d(app)}$ will be within a few orders of magnitude of the IC_{50} we can use K_p to estimate the value of $K_d{}'$, and find this to be ~100 μM. This value would represent the equilibrium dissociation constant between the lipid partitioned ligand and the lipid embedded receptor. Remarkably, however, the value is in good agreement with equilibrium dissociation constants measured between micelle embedded receptors and free ligands [48, 55, 64].

Considering the differences in the models used to derive these values, and the incomplete understanding of the structure of the ligand-receptor complex, it is difficult to draw any firm conclusions from this, other than to note that lipid partitioning of these ligands will have a significant effect on $K_{d(app)}$ and $IC_{50}$and that accurate measurements of $K_p$in an appropriate lipid model will be important in interpreting functional results. In measurements of K_p in such studies, it is imperative that the same lipid composition is maintained for both the cell-based functional assay and the K_p measurement in solution (see also 3.3 on cell dependent lipid compositions). For example, in studies of dihydropyridines, a calcium channel antagonist, [74, 75] and dopmaine receptor antagonists [76] the K_p was significantly affected by the cholesterol content and phase of the membrane (liquid crystalline *vs* gel state).

Currently, K_p values are derived using artificial membranes composed of purified lipids, assembled into large unilamellar vescicles (LUVs), where peptide binding is quantitated using a combination of sedimentation, chromatographic and fluorescence based techniques [71]. There are two disadvantages with this approach, first the lipid bilayer will not have the same composition as that used functional assays, and second, the sedimentation and fluorescence will favour lipid insertion over weak electrostatic interactions that may also lead to membrane accumulation. An approach that may address some of these problems is the use of lipid nanodiscs [77-79]. These are disc shaped soluble particles consisting of a flat lipid bilayer enclosed by a membrane scaffolding protein (MSP). Thus, lipid extracts from cells can be directly assembled into the nanodiscs and these can be used in more sensitive and higher resolution biophysical assays such as nuclear magnetic resonance (NMR) and surface plasmon resonance (SPR) experiments. Although we note that any interaction between the ligand and the MSP itself must be controlled for carefully. The former is of particular interest as the use of isotope labelling allows for both structural and functional studies. There have been significant developments in application of multidimensional NMR experiments to venom peptides as well as structural studies of membrane proteins in nanodiscs [78, 80, 81]. A

combination of these technologies is likely to provide new insights into the mechanism of membrane binding of GMTs.

3.3 Lipid composition

In the preceding sections, we found that membrane association is a significant component of the apparent affinity of many VGIC ligands. We also know that the association is highly dependent on the exact lipid composition. In this section, we will briefly describe both the lipids themselves and their composition in relevant cell types. This is particularly important in the development of biophysical assays of lipid binding (K_p) that would be relevant to correlate to functional data (IC_{50}), as discussed before, but also for interpretation of emerging structural data.

Lipids make up the bulk of the cellular membrane. The three main types of lipids are phospholipids, glycolipids, and cholesterol, with phospholipids being the major component of the membrane. Phospholipids derived from glycerol are called phosphoglycerides (Fig. 5A) while those from sphingosine are called sphingomyelins or glycolipids (Fig. 5B). Glycolipids differ from sphingomyelin in the moiety that is attached to the primary OH group of the sphingomyelin backbone: one or more sugars in glycolipids versus phosphoryl choline in sphingomyelin (*c.f.* Fig. 5B). Cholesterol differs from all these lipids in that it is a steroid type lipid (Fig. 5C). Cholesterol is absent in prokaryote cells whereas it can be present at a very high concentration in animal membrane [82].

[Figure 5 near here]

The composition of the cell membranes is dependent on a number of factors including the cell type. Considering that different cell types are used in VGICs functional assays it is important to consider what the lipid composition is in the membrane of these cells. The majority of VGIC cell-based assays either use channels that are transfected into immortalised cells or neuronal cells that are selected for overexpression of a particular ion channel and then immortalised. The primary cell types used in functional assays are mammalian neuronal cells, HEK cells and CHO cells as well as amphibian oocytes. In Table 5 we have provided an overview of the major lipid content of these cell types. A wealth of other lipids is present but each constituting < 5% of the total lipid content and therefore unlikely to contribute significantly to the K_p of GMTs and are therefore excluded. It should be noted that the data presented is from different sources (sometimes using different methods) and should be considered a very crude generalisation. What should also be considered is that the lipid composition of a cells will be different dependent on the metabolism of the cell (affected by the growth media and conditions as well as the point in the cell cycle) and finally, it may be that the VGICs are associated in multiprotein complexes in lipid rafts that themselves may have very different lipid composition compared to the rest of the plasma membrane. Nevertheless, the table points to a general composition of ~50-60% of PC/PE lipids 30-40% cholesterol and about 5% each of sphingomyelin (SM), phosphatidylserine (PS) and phosphatidylinositol (PI) lipids. Of these PS is of particular interest as it is anionic and would in a similar manner to PG attract the cationic peptides, however, PS is enriched in the inner leaflet of the membrane and is therefore not likely to have a significant effect on peptide activity [83]. Similarly, PI is largely found on the inner leaflet and is unlikely to make a have a significant contribution to peptide binding [83]. In contrast SM is enriched in the outer membrane and may be present in a higher abundance than indicated

by Table 5 in the relevant region of the cell membrane for GMT binding. Thus, a model membrane containing 60% POPC, 30% cholesterol and 10% SM would appear to be reasonable membrane composition for measurement of K_p values for GMTs, for comparison with data from cell-based assays.

[Table 5 near here]

Much of the VGIC structural data to date has been from prokaryotes, first from archea and later from bacterial sources, each with their own unique lipids. The membranes of archaea are distinguished from those of eukaryote or bacteria in several ways. First, the two nonpolar chains (alkyl chains) are joined to the glycerol backbone through an ether bond instead of an ester bond (the dashed rectangle in Fig. 6) – making it less polar. Second, the alkyl chains are branched with methyl groups rather than being linear (the dashed ellipsoid in Fig. 6) – making these more hydrophobic and potentially affecting the lipid order. Third, the glycerol backbone is a stereoisomer of that in phosphatides (Figs 5A and 6) – providing a different chirality [84], and finally, the phosphate headgroups are decorated with different sugar moieties. Thus, studies of archeal bacterial channels such as KvAP in POPC and POPG (prevalent in bacteria) is likely to have consequences for the equilibrium population of the different states of the channel in the presence and absence of membrane voltage. An interesting observation is that VSTx1, a peptide that binds to the inactivated state of KvAP in POPE/POPG mixtures is also a weak inhibitor of Na_V channels [85], most likely by binding to the down state of these channels (it contains 6 of the 7 conserved residues in the HwTx-IV family of peptides as shown in Table 4). This would suggest that the up state of the VSD of KvAP in a POPE/POPG mix shares structural homology to the down state of Na_V channels and the mechanism of voltage gating of K_V channels in archea may indeed be rather different to that observed in mammalian cells considering the divergent lipid composition of the lipid bilayer as well as the significant structural changes involving loop sizes and the helix break in KvAP [86].

[Figure 6 near here]

4. Concluding remarks

VGICs are important pharmacological targets. The allosteric modulation of these channels through their VSDs presents an opportunity for development of state and subtype selective modulators. In principle, this mode of channel modulation would address many neurological disorders but remains largely unexplored. To date, the most abundant source of gating modifiers has come from peptide toxins from animal venoms. In this review, we have shown that with detailed knowledge of the VSD architecture and careful structure-activity data it is possible to use bioinformatic tools to analyse complex sequence data to generate novel peptide screening tools and identify potential off-targets. This bioinformatic screening approach is very attractive in the context of the wealth of sequence information that is being generated but also hinges on availability of high quality experimental data, further highlighting the need for efforts to characterise the structural mechanism of channel function and inhibition. Such studies are hampered by the integral role of the membrane in channel inhibition by gating modifiers. However, recent advances in development of membrane mimetics such as nanodiscs combined with biophysical characterisation by NMR and cryo-EM promises to lead to new advances in our understanding of VGICs and ultimately in realising the great potential of this class of molecules as pharmacological targets.

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Competing interests statement

The authors have no competing interests to declare.

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Tables

Table 1. Proteins identified as being part of the voltage-gated ion channel family by the International Union of Basic & Clinical Pharmacology (IUPHAR) [21].

	Transmembrane (TM) segments [TMs per subunit]	Voltage sensor domain?	Ligand gated	Voltage sensing protein?
Na_V	***24 [24]***	***Yes***	***No***	***Y***
K_V	***24 [6]***	***Yes***	***No***	***Y***
Ca_V	***24 [24]***	***Yes***	***No***	***Y***
CatSper	***24 [6]***	***Yes***	***No***	***Y***
TP-channels	***24 [12]***	***Yes***	***No (sensitive to $PIP_{3,5}$)***	***Y***
H_V channels	***8 [4]***	***Yes***	***No (highly pH sensitive)***	***Y***
CNG/HCN	***24 [6]***	***Yes***	***Yes (cGMP/cAMP)***	***Y***
K_{Ca}	24 [6]	Yes	Yes (Ca^{2+})	N
TRP channels	24 [24]	Yes	Yes (various)	N
K_{2P}	8 [4]	No	Yes (neurotransmitters)	N
K_{ir}	8 [2]	No	Yes (PIP_2)	N

Table 2. Putative voltage-sensing human proteins identified based on sequence alignment templated of Palovcak et al. [22]. These are proteins identified as having a voltage sensor domain (VSD) and are here further classified based on the number of gating charges in their VSDs. Where channels have more than one VSD, the VSD with the largest number of gating charges is used. Channels having a VSD with ≥ 4 gating charges are given in standard font in black. Channels with VSDs containing 3 gating charges are shown in green with italic font, those containing 2 gating charges are shown in orange and bold font and those containing no gating charges are shown in red with bold and italic font.

CatSper	Ca_V	HCN/CNG	K_V			Na_V	Other
Catsper1	$Ca_V1.1$	HCN1	$K_V1.1$	$K_V4.1$	$K_V8.1$	$Na_V1.1$	Nax
Catsper2	$Ca_V1.2$	HCN2	$K_V1.2$	$K_V4.2$	$K_V8.2$	$Na_V1.2$	*H_V1*
Catsper3	$Ca_V1.3$	HCN3	$K_V1.3$	$K_V4.3$	$K_V9.1$	$Na_V1.3$	*TM266*
Catsper4	$Ca_V1.4$	HCN4	$K_V1.4$	$K_V5.1$	$K_V9.2$	$Na_V1.4$	*NalCN*
	$Ca_V2.1$	*CNG4*	$K_V1.5$	*$K_V6.1$*	$K_V9.3$	$Na_V1.5$	**TPTE**
	$Ca_V2.2$		$K_V1.6$	*$K_V6.2$*	$K_V10.1$	$Na_V1.6$	**TPTE2**
	$Ca_V2.3$		$K_V1.7$	*$K_V6.3$*	$K_V10.2$	$Na_V1.7$	**$K_{Ca}5$**
	$Ca_V3.1$		$K_V1.8$	*$K_V6.4$*	$K_V11.1$	$Na_V1.8$	**NHE11**
	$Ca_V3.2$		$K_V2.1$	**$K_V7.1$**	$K_V11.2$	$Na_V1.9$	*TPC1*
	$Ca_V3.3$		$K_V2.2$	*$K_V7.2$*	$K_V11.3$		*TPC2*
			$K_V3.1$	*$K_V7.3$*	$K_V12.1$		***PKD2L1***
			$K_V3.2$	*$K_V7.4$*	$K_V12.2$		***$K_{Ca}1.1$***
			$K_V3.3$	*$K_V7.5$*	$K_V12.3$		
			$K_V3.4$				

Table 3. Human VGICs are shown with VSDs that contain conserved residues that confer sensitive to HwTx-IV (aligned based on the described HMMER profile). Five residues were shown to be important for HwTx-IV binding to the VSD of Na_Vs. The conservation of these residues in other channels is shown below. Those in bold have all of the required residues and are most likely to be sensitive to HwTx-IV (indeed both $Na_V1.7$ and $Na_V1.6$ have been shown to be inhibited potently by this toxin). The alignment is available in the supplemental material section (Figure S1).

VGIC	Sequence	# Required residues	VGIC	Sequence	# Required residues
$Na_V1.7$	**EELDE**	**5**	$K_V1.1$	ETTIE	2
$Na_V1.6$	**EELDE**	**5**	$K_V1.2$	ETTLE	2
$Ca_V1.3$	**EELEE**	**5**	Kv1.3	ETTLE	2
$Na_V1.1$	*EELNE*	*4*	$K_V1.5$	ETTLE	2
$Na_V1.2$	*EELNE*	*4*	$K_V4.3$	EGMNE	2
$Na_V1.3$	*EELNE*	*4*	$K_V9.3$	HTVTE	2
$Ca_V1.1$	*QDLED*	*4*	NALCN	NSLVE	2
$Ca_V3.1$	*EGLEE*	*4*	Nav NAX	ESGTE	2
$Ca_V3.2$	*EGLEE*	*4*	$K_V10.1$	NIFND	1
$Ca_V3.3$	*EGLEE*	*4*	$K_V10.2$	NI-AE	1
Nav1.9	ESITE	3	$K_V11.1$	SLGGE	1
$K_V4.1$	EGVKD	3	$K_V11.3$	SLGGD	1
$K_V4.2$	EGMDE	3			
HCN3	GIVEE	3			
TPTE2	DDYFD	3			
TPTE	DVVFD	3			

Table 4. Toxins predicted by our gating modifier HMMER profile to bind to the voltage-sensing domain (VSD) of $Na_V1.7$. The Uniprot ID of each hit is reported as well as the number of required residues and the sequence of these residues - footnotes are provided for information available in UniProt regarding off-targets. The alignment is available in the supplemental material section (Figure S2).

Toxin	Uniprot ID	# Required residues	Sequence
Mu-theraphotoxin-Hs2a	P83303	7	IFSWCKY
Mu/omega-theraphotoxin-Hs1a	P56676	7	VFSWCKW
Beta/kappa-theraphotoxin-Hlv1a	B3EWN3[a]	7	LFSWCKY
Beta-theraphotoxin-Ps1a	P84510	7	FLSWCKY
Omega-theraphotoxin-Gr2a	P0DJA9	7	FMSWCKY
Beta-theraphotoxin-Cm1a	P84507[b]	7	WFSWCKY
Beta-theraphotoxin-Cm1b	P84508[b]	7	WFSWCKY
Mu-theraphotoxin-Hhn1a	P60975	6	FGSWCKY
Beta-theraphotoxin-Hlv1a	B3EWN2	6	FGSWCKY
Kappa-theraphotoxin-Tb1a	P83745[c]	6	MFNWCKY
Mu-theraphotoxin-Hhn2a	D2Y2D1	6	FGSWCKV
Kappa-theraphotoxin-Tb1c	P83747[c]	6	MFRWCKY
Kappa-theraphotoxin-Tb1b	P83746[c]	6	MFRWCKY
Beta-theraphotoxin-Pmr1a	B3EWN0	6	MFNWCKY
Kappa-theraphotoxin-Gr4a	P0C2P5[d]	6	WFNWCKY
Jingzhaotoxin F7	P0CH54	6	PFSWCKY
U24-theraphotoxin-Cg1a	B1P1H2	6	LFEWCKI
Kappa-sparatoxin-Hv1d	P61792[e]	6	LFRWCKY
Kappa-sparatoxin-Hv1e	P61791[e]	6	LFHWCKY
Mu-theraphotoxin-Hhn2b	D2Y1X6	5	FGNWCKV
Mu-theraphotoxin-Tp1a	P0DL64[f]	5	FGGWCKL

a. Blocks human (IC_{50}=80 nM) and rat (IC_{50}=160 nM) $Na_V1.3$, partially inhibits human $K_V11.1$/KCNH2/ERG (25% at 175 μM), has no effect on human or rat $Na_V1.8$.

b. Inhibits $Ca_V2.2$ but does not inhibit $Ca_V1.3$ or $Ca_V3.1$.

c. Blocks $K_V4.2$ channels (Tb1a: IC_{50} is 193.0 nM); Tb1b is a low affinity blocker of $K_V4.2$.

d. Weakly inhibits K_VAP.

e. Inhibitor of the K_V4/KCND family, also blocks calcium channels (Ca_Vs).

f. Affects neither human Ca_Vs nor nicotinic acetylcholine receptors (nAChR) at 5 μM.

Table 5. Estimates of relative abundance of the major lipid components of four cell types typically used in functional assays of voltage-gated ion channels. The values in the table are normalised assuming they only contain these major components and are given in weight percent unless otherwise indicated. Data are for cells at a stationary phase where available.

Cell type	Membrane lipids					
	CHL	PC	PE	PS	SM	PI
Synaptic vesicle [87]	29%	26%	30%[*]	9%	5%	1%
CHO cells [88, 89]	26%[#]	25%	36%	5%	5%	2%
HEK cells [89, 90]	19%	28%	37%	5%	6%	4%
Xenopus oocytes [91, 92]	39%[#]	40%	11%	1%	3%	6%

Abbreviations: CHL-cholesterol, PC-phosphatidylcholine, PE-phosphatidylethanolamine, PS-phosphatidylserine, SM-sphingomyelin, PI-phosphatidylinositol, PL = phospholipid.

[#] *Mole percent.*

[*] *The content of PE given as sum of 1-ester (17%) and 1-ether (13%) content.*

Figures:

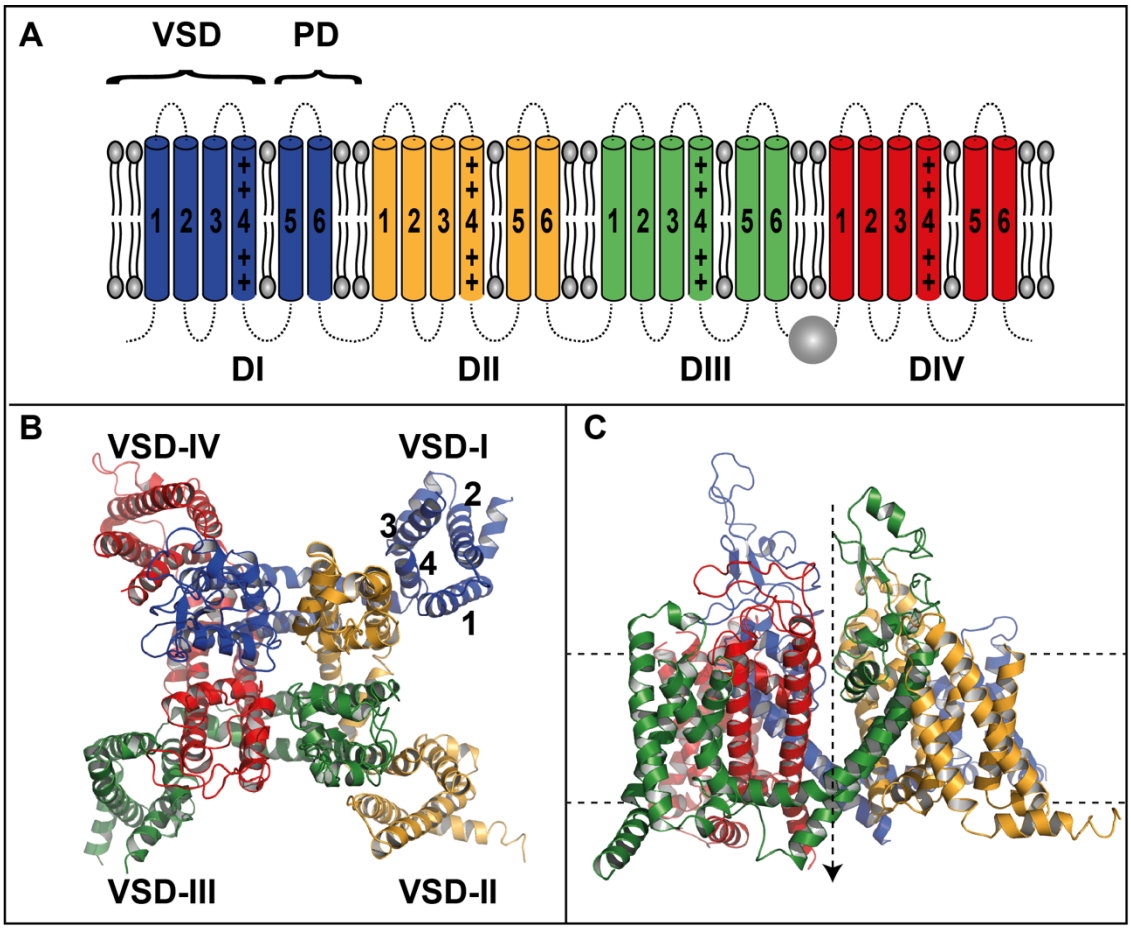

Figure 1. Architecture of voltage-gated ion channels (VGICs). A) Schematic image of the six transmembrane helices (S1-S6) of each of the four domains (DI-DIV) of a VGIC. The linkers between the helices are dotted as they vary significantly in length depending type of VGIC and the interdomain linkers are absent in tetrameric channels. The voltage sensing domain (VSD) and the pore domain (PD) of the first domain are annotated. The inactivation (IFM) particle of Na_Vs in the DIII-DIV linker is depicted as a ball. The six helices of each of the four domains come together to form the functional channel. B) Top view a cartoon model of a recently described [20] mammalian voltage gated sodium channel ($Na_V1.4$). The four domains are annotated and the four helices making up the VSD of domain I are also annotated. The ion-conducting pore is at the hollow centre of the channel. C) Side view of the channel shown in B, where the large extracellular loops of the channel are evident. The approximate position of the lipid bilayer is indicated by the dotted lines. The ion-conduction path is indicated by the dotted arrow.

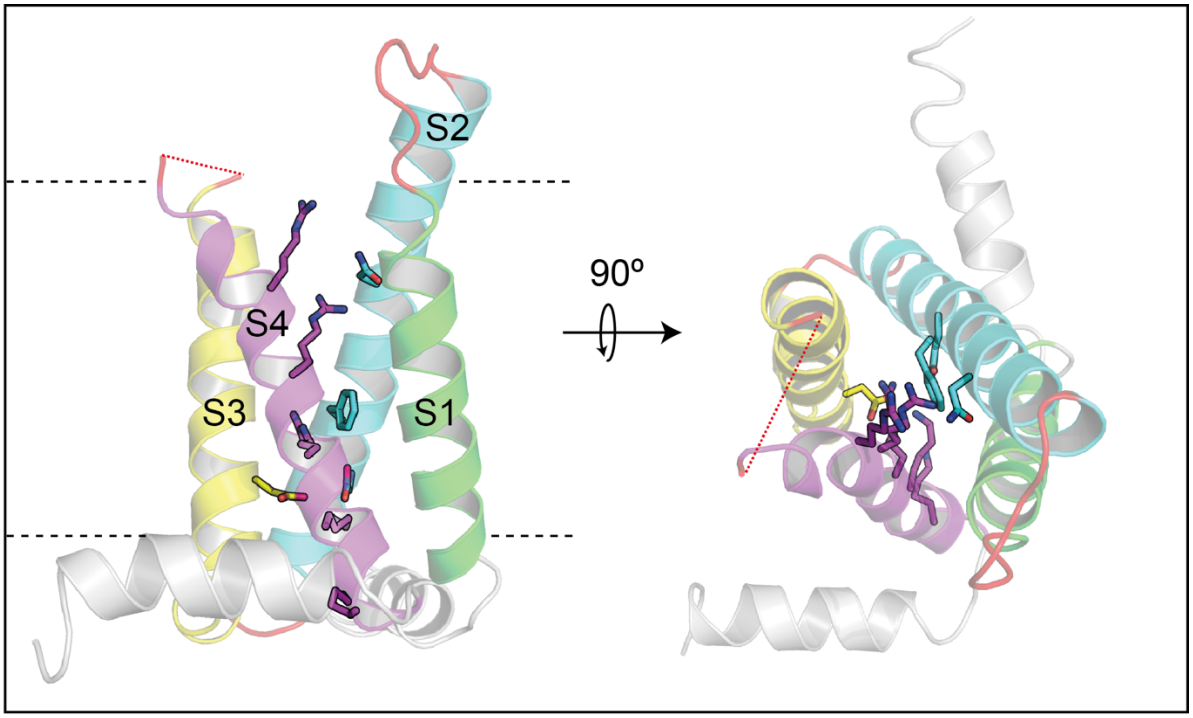

Figure 2. Figure showing the conserved amino acids in the S1-S4 helices of the voltage sensing domain (VSD) of voltage-gated ion channels (VGICs). The S3-S4 loop is missing in the PDB structure used - the missing linker is indicated by a dotted line [20]. The sidechains of the positively charged "gating" residues (arginines and lysines) on S4 are shown. The sidechains of conserved residues on S2 and S3 are also shown, which include the counter-charged (acidic) residues on S2/S3 and the hydrophobic plug (phenyl alanine) on S2. Movement of the S4 helix, in response to changes in membrane voltage, is thought to open and close the pore domain.

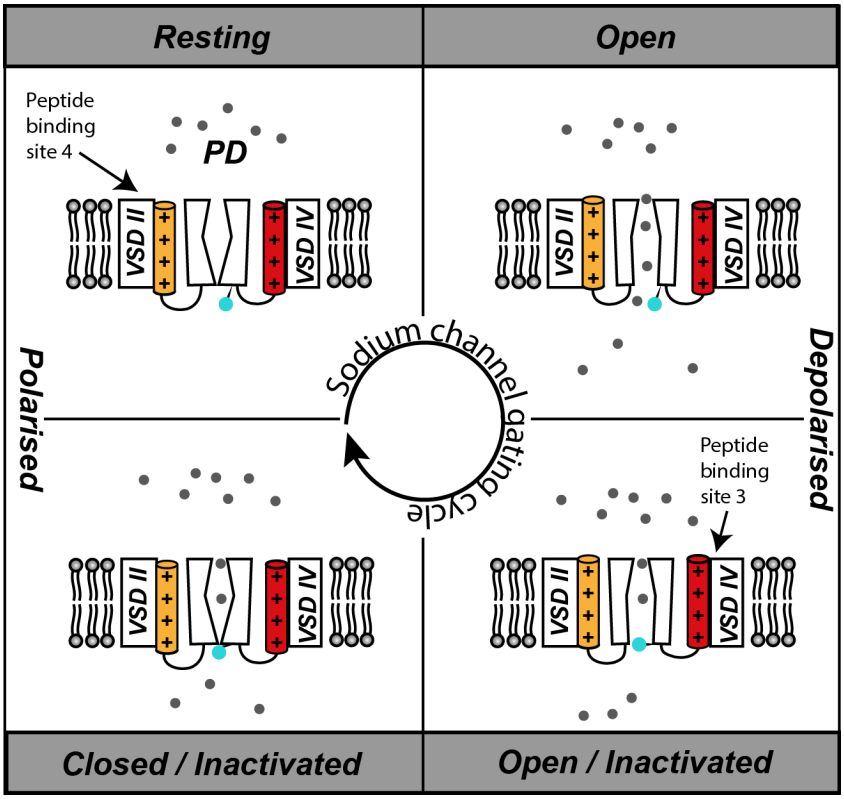

Figure 3. Schematic of a simplified model of the gating cycle in voltage gated sodium channels ($Na_V s$). Only voltage sensing domains (VSDs) of domains II and IV are shown. The 'up' and 'down' states are indicated by movements of the gating charges in the VSD (cylinder containing four '+' symbols) with relation to the lipid bilayer. The fast inactivation of the channel by the inactivation (IFM) particle (cyan ball below the pore) is also shown. The binding site of venom peptides that can inhibit the channel are indicated with arrows, in the state that they would lead to inhibition of ion conduction. Upon membrane depolarisation, movement of the gating charges of the VSD open the inner gate of the pore domain and the channel transitions from the resting state (top left) to the open state (top right). The movement of the DIV gating charges lead to channel inactivation (bottom right), where the inner gate is open but occluded by the IFM particle. Upon repolarisation the gating charges move towards the intracellular side of the membrane and the inner gate closes and the channel is found in a closed inactivated (or deactivated) state (bottom left). Movement of the inactivation particle then returns the channel to the resting state.

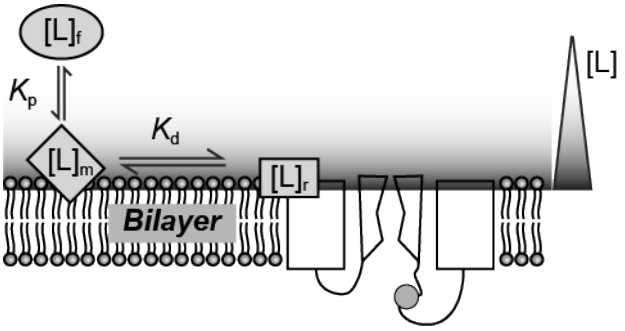

Figure 4. Schematic description of the how gating modifier peptides bind to voltage-gated ion channels. The peptide in solution (L_f) first associates with the membrane (L_m), increasing its concentration near the receptor, prior to receptor binding (L_r).

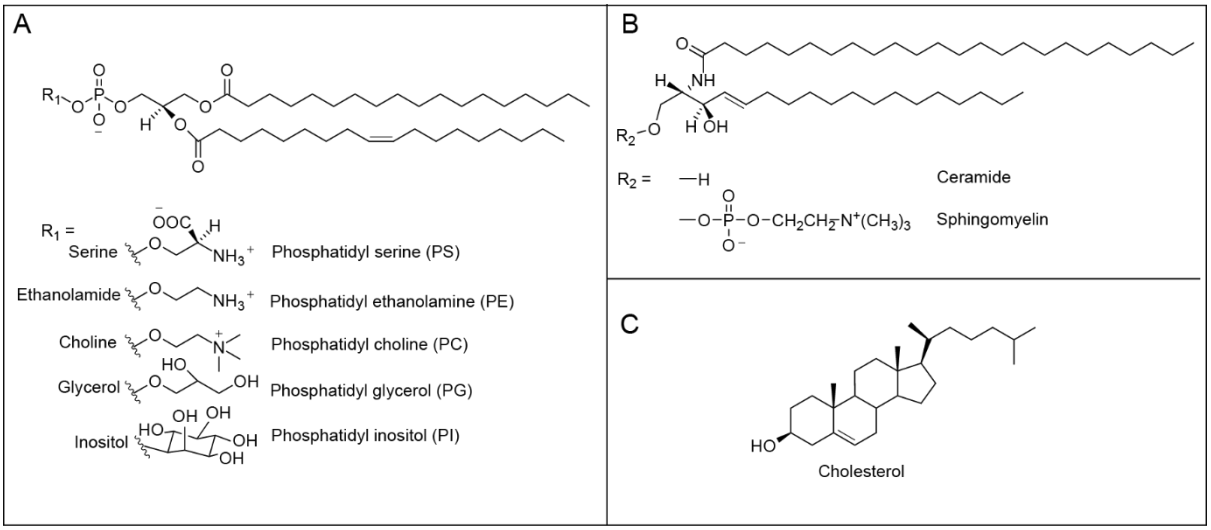

Figure 5. Chemical structures of A) common phosphoglycerides, B) a ceramide and sphingomyelin and C) cholesterol.

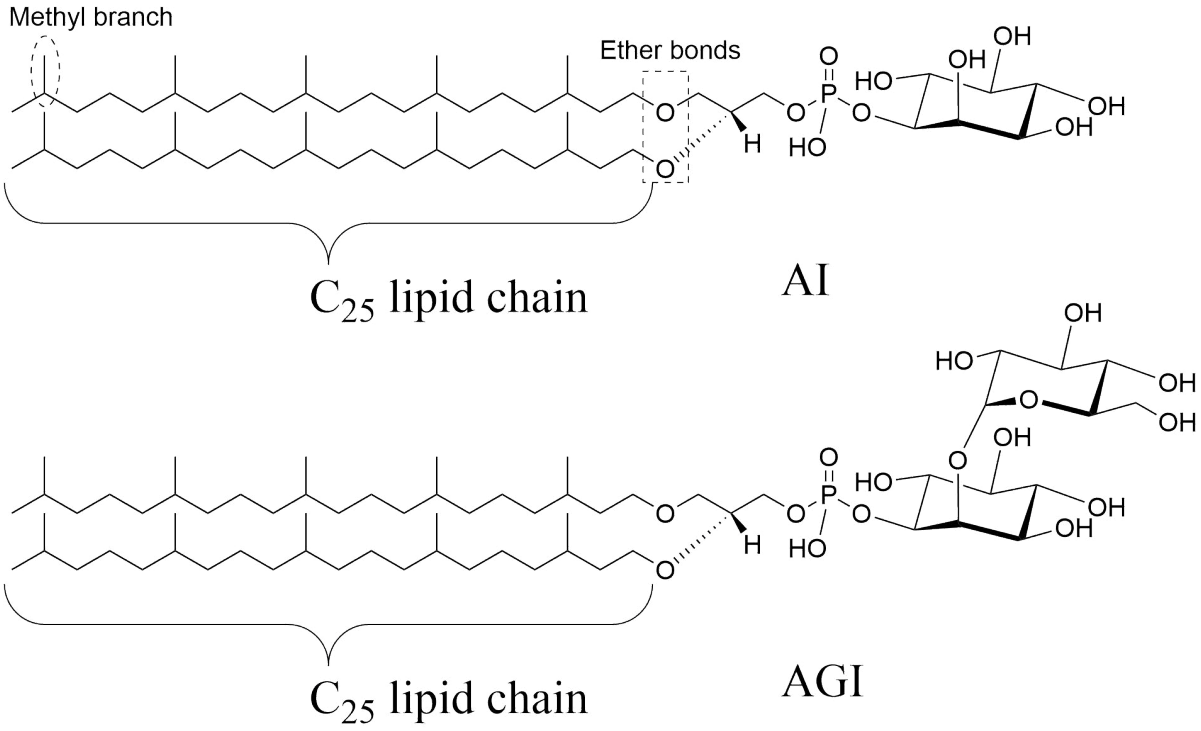

Figure 6. The structural formula of 2,3-di-O-sesterpanyl-sn-glycerol-1-phospho-myo-inositol (C 25,25 -archetidylinositol) (top: AI) and 2,3-di-O-sesterpanyl-sn-glycerol-1-phospho-1'-(2'O-α-D-glucosyl)-myo-inositol (C 25,25 -archetidyl (glucosyl) inositol) (bottom: AGI). The C25 lipid chain, methyl branch and ether bonds are annotated. AGI and AI were the only two major lipids, accounting for 91 mol% and 9 mol% of total polar lipids, of Aeropyrum pernix *respectively [93, 94].*