

WHITE PAPER

Na⁺ channel function, regulation, structure, trafficking and sequestration

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Abstract This paper is the second of a series of three reviews published in this issue resulting from the University of California Davis Cardiovascular Symposium 2014: *Systems approach to understanding cardiac excitation–contraction coupling and arrhythmias: Na⁺ channel and Na⁺ transport*. The goal of the symposium was to bring together experts in the field to discuss points of consensus and controversy on the topic of sodium in the heart. The present review focuses on Na⁺ channel function and regulation, Na⁺ channel structure and function, and Na⁺ channel trafficking, sequestration and complexing.

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Abbreviations AP, action potential; CaM, calmodulin; CaMKII, CaM-dependent protein kinase II; Cx43, connexin43; EAD, early afterdepolarization; E_m , membrane potential; I_{Na} , Na⁺ current; I_{NaL} , the late Na⁺ current; LQT3, long QT syndrome type 3; Na_v channel, voltage-gated sodium channel; PD, pore-forming domain; VSD, voltage-sensing domain.

Na⁺ channel function and regulation

In the session on 'Na⁺ channel function and regulation', presentations were given by Hugues Abriel, Ye Chen-Izu, Victor

Maltsev and Thomas Hund. Geoffrey Pitt led the discussion with panelists Eleonora Grandi, Randall Rasmusson, Lars Maier and Celine Marionneau. Below is a summary of

the salient points and perspectives on the major issues and controversies in the field.

Voltage-gated sodium channels (Na_V) are responsible for initiation and propagation of the action potential (AP) in nerve, muscle and heart, where they are crucial determinants of many aspects of excitability and contraction. During the upstroke (phase 0) of the cardiac AP, cardiac Na_V channels open rapidly, creating a large spike of inward Na^+ current (I_{Na}) and then quickly transition into a non-conducting inactivated state. This inactivated state renders cardiomyocytes resistant to immediately firing a second AP, until the channels recover from inactivation (which requires time and negative membrane potential, E_m). The activation and inactivation processes ensure the appropriate temporal and directional spread of electrical activity throughout the myocardium for the coordinated contraction necessary to propel blood throughout the body. While Na^+ channels are in the inactivated state, cardiomyocytes are relatively protected from initiating afterdepolarizations and out-of-rhythm APs, thereby limiting electrical activity that may trigger arrhythmias. While the process above describes the situation for the majority of Na^+ channels, there is a small probability that a fraction of channels do not remain inactivated, allowing them to re-open and/or stay open during the repolarization phase of the AP. The Na^+ current resulting from these events is called the 'late' Na^+ current (I_{NaL}) or, alternatively, the 'persistent' or 'sustained' current (Maltsev *et al.* 1998) and is often distinguished as a separate component of I_{Na} . I_{NaL} has received increasing attention since it has been shown that selective inhibition of this current could prevent the emergence of arrhythmogenic APs (Maltsev *et al.* 2007; Zaza *et al.* 2008; Moreno & Clancy, 2012; Yang *et al.* 2012; Shryock *et al.* 2013).

The late Na^+ current (I_{NaL}). A description of the physiological and pathophysiological roles of I_{NaL} has been a major area of interest for many years, as I_{NaL} is significantly increased in pathophysiological states such as heart failure and it is tightly linked to arrhythmias (Undrovinas *et al.* 2002; Valdivia *et al.* 2005; Maltsev *et al.* 2007; Shryock *et al.* 2013; Antzelevitch *et al.* 2014). Gain-of-function mutations in *SCN5A* that encodes the major cardiac Na^+ channel $\text{Na}_V1.5$, cause long QT syndrome type 3 (LQT3) by increasing I_{NaL} resulting in cardiac AP prolongation that could act as a substrate for arrhythmias (Bennett *et al.* 1995; Rivolta *et al.* 2001). However, what produces I_{NaL} and how it is regulated at the molecular level in cardiomyocytes are not fully understood. I_{NaL} can clearly be measured in the major cardiac $\text{Na}_V1.5$ channel, even in heterologous systems; β subunits seem to be required for the effect (Mishra *et al.* 2011). However, specific inhibition of the $\text{Na}_V1.8$ (*SCN10A*), which is not a major

contributor to peak I_{Na} in cardiac myocytes, has been shown to reduce cardiac I_{NaL} (Yang *et al.* 2012); so even the identity of the key channel subunits for I_{NaL} is incompletely resolved. Multiple attempts to dissect the identity of I_{NaL} in cardiomyocytes have been undertaken via electrophysiological and biophysical experimental techniques as well as by computational modelling approaches. I_{NaL} (Maltsev & Undrovinas, 2006) has been investigated in detail, including the contribution of the current to different arrhythmia mechanisms (early *vs.* delayed afterdepolarizations) (Undrovinas *et al.* 2002), its role in heart failure (Valdivia *et al.* 2005; Maltsev *et al.* 2007), and how various Na^+ channel-associated proteins affect the development of I_{NaL} .

Question or controversy: the late Na^+ current

What are the mechanisms of I_{NaL} ?

How is I_{NaL} regulated in normal cardiac myocytes and in disease states?

Most studies of I_{NaL} in both myocytes and heterologous expression systems have assessed I_{NaL} using classical square pulse depolarizing step protocols as in Fig. 1A, and non-physiological ionic conditions to isolate I_{NaL} (Maltsev *et al.* 2007; Maltsev & Undrovinas, 2008). While such experimental approaches have been highly informative, the actual I_{NaL} may be quite different in a myocyte that experiences an AP with dynamic changes in E_m , intracellular $[\text{Ca}^{2+}]$ and contraction under physiological conditions. Moreover, Na^+ channels in cardiomyocytes are also anchored by large macromolecular complexes and are thus interacting with many regulatory proteins not present in heterologous expression systems (Nerbonne & Kass, 2005; Shy *et al.* 2013).

Figure 1B shows measurements of I_{NaL} in ventricular cardiomyocytes during the cardiac AP using a novel self-AP-clamp technique (Banyasz *et al.* 2011; Chen-Izu *et al.* 2012; Horvath *et al.* 2013). This technique uses the myocyte's own characteristic AP as a voltage-clamp waveform measured under physiological conditions, with preserved Na^+ and Ca^{2+} homeostasis. Then one can sequentially block specific currents such as I_{NaL} (e.g. with TTX, or more I_{NaL} -selective compounds like GS458967; Belardinelli *et al.* 2013). The difference current can be used to infer the actual I_{NaL} flowing during the physiological AP. One can then, in the same myocyte, assess K^+ currents the same way, so that in the case shown in Fig. 2B one can measure the rapid and slow delayed rectifier, and inward rectifying, K^+ currents (I_{Kr} , I_{Ks} and I_{K1}) to assess the balance of key inward and outward currents during the AP plateau. With respect to I_{NaL} , two things are apparent and differ from classical square pulse measurements. First, the I_{NaL} amplitude appears to be larger than expected, and is comparable in scale to K^+ currents during the plateau. Second, I_{NaL}

is relatively clearly separated from the fast peak I_{Na} , and progressively increases as repolarization proceeds during the plateau, because the driving force ($V_m - E_{Na}$), the difference between the membrane potential and the Na⁺ equilibrium potential, is increasing faster than conductance is declining. This differs dramatically from the monotonic I_{NaL} decline seen in square pulses and makes it more apparent how I_{NaL} can contribute to early afterdepolarizations (EADs).

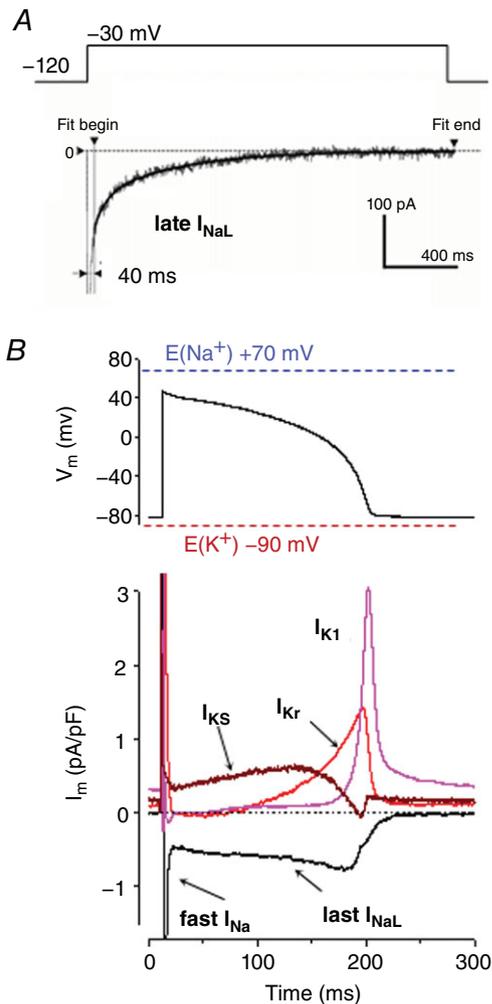


Figure 1. Late Na⁺ current in ventricular myocytes

A, I_{NaL} recorded in a human ventricular myocyte using traditional square voltage clamp pulse from -120 mV (where Na⁺ channel availability is maximized) to -30 mV (where the peak of I_{Na} occurs; from Maltsev & Undrovinas, 2006, with permission). B, I_{NaL} recorded under ^{self}AP-clamp in guinea pig ventricular myocyte (from Horvath *et al.* 2013, with permission). Notice that the I_{NaL} during AP can be clearly separated from the fast Na⁺ current at the onset of AP. I_{NaL} is sustained during the AP plateau phase, and increases as repolarization proceeds. This is because the driving force ($E_m - E_{Na}$) increases faster than the conductance declines during this phase. Nernst potentials for Na⁺ and K⁺ are shown (E_{Na} and E_K). I_{NaL} and K⁺ currents (I_{Kr} , I_{Ks} and I_{K1}) can be recorded from the same cell under ^{self}AP-clamp Sequential Dissection.

Another limitation of the square pulse voltage-clamp protocols to measure I_{NaL} is that there is no unambiguous separation between the peak and the late components in the monotonically decaying current trace (Maltsev & Undrovinas, 2006). A commonly accepted practice is to fit I_{NaL} after a somewhat arbitrary delay (e.g. 40–50 ms after the onset of depolarization with currents recorded at room temperature). While practical, that approach is likely to underestimate I_{NaL} and provide limited kinetic information with respect to function during the AP. Another complication in measuring I_{NaL} relates to a form of Na⁺ channel gating called *non-equilibrium gating*. Clancy *et al.* (2003) first discovered an enhancement of I_{Na} under a repolarizing voltage ramp (non-equilibrium condition), which is not seen under square pulse protocols. This phenomenon can be explained by a fast recovery of the Na⁺ channel from the inactivated state and reactivation during the repolarization phase. The non-equilibrium gating may give rise to a substantial portion of I_{NaL} and, importantly, this portion of the current cannot be activated by using a square pulse voltage-clamp protocol (Clancy *et al.* 2003; Moreno & Clancy, 2012). A recent study also demonstrated how this non-equilibrium gating of Na⁺ channels can be responsible for EADs in mouse ventricular myocytes which have a late plateau phase at E_m more negative than -45 mV (that is driven by large Ca²⁺ transients and Na⁺/Ca²⁺ exchange current) (Edwards *et al.* 2014). This differs from EAD mechanisms that occur at positive plateau E_m seen in human, rabbit or guinea-pig ventricle, but might be relevant to some human atrial myocytes that have a similar waveform.

In summary, recent studies suggest that the magnitude of I_{NaL} is much larger than previously appreciated. Moreover, I_{NaL} is increased in heart failure and is thus probably a major contributor to the AP prolongation and the increased arrhythmia propensity that accompanies heart failure (Shryock *et al.* 2013). It will be highly informative to use the ^{self}AP-clamp Sequential Dissection technique to record I_{NaL} under different pathophysiological conditions, such as experimental models of long QT syndrome and heart failure.

Question or controversy: the late Na⁺ current

What is the magnitude of I_{NaL} under physiologically relevant conditions?

How to capture all the possible mechanisms contributing to I_{NaL} in experimental measurements?

Na⁺ channel regulation by Ca²⁺, calmodulin and calmodulin-dependent protein kinase II. An important component of Na⁺ channel regulation is by the Ca²⁺, calmodulin (CaM) and CaM-dependent protein kinase II (CaMKII) pathway that affects channel function. Ca²⁺ is

found to modulate Na⁺ channel inactivation (Tan *et al.* 2002; Sarhan *et al.* 2012). CaM is known to bind to the Na⁺ channel IQ domain in both Ca²⁺-bound and Ca²⁺-free forms (Bahler & Rhoads, 2002; Shah *et al.* 2006). The mechanisms of Ca²⁺ and CaM regulation and their collective effects on Na⁺ channel has been a subject of extensive study in the field, which was summarized in a recent review by Van Petegem, Lobo and Ahern (Van Petegem *et al.* 2012). They proposed that Ca²⁺ modulation of I_{Na} facilitation occurs at high pacing frequency when Ca²⁺ levels accumulate. Many Na⁺ channel disease mutations are located in Ca²⁺-sensing domains, and disruptions of Ca²⁺-dependent regulation contribute to Na⁺ channel dysfunction.

Wagner *et al.* (2006) first showed that CaMKII interacts with, and phosphorylates, the cardiac Na⁺ channel, and causes major changes in I_{Na} gating and [Na⁺]_i in rabbit and mouse ventricular myocytes. They showed that CaMKII caused a negative shift in Na⁺ channel availability (steady state inactivation), but no change in activation E_m dependence or peak amplitude of fully available I_{Na} . There was also an increase in intermediate inactivation (but no change in rapid inactivation) and slowed recovery from inactivation. These changes are all loss-of-function effects that would reduce I_{Na} in the heart (analogous to certain genetic short QT or Brugada mutations). However, CaMKII also greatly increased I_{NaL} , which would be a gain-of-function and mimic LQT3. Interestingly, the array of CaMKII-induced alterations in I_{Na} gating almost exactly phenocopies the effects of a human point mutation in Na_v1.5 (1795InsD) that is associated with both LQT and Brugada syndromes, depending on heart rate (Veldkamp *et al.* 2000). Since CaMKII is known to be upregulated and more active in heart failure this could be an acquired form of arrhythmias in heart failure patients (Erickson & Anderson, 2008; Bers & Grandi, 2009; Anderson *et al.* 2011). The elevated CaMKII activity in heart failure and other pathologies can also modulate many ion channels and Ca²⁺ transport proteins, including I_{Na} , I_{Ca} , I_{to} , I_{K1} , $I_{K(ATP)}$ and the ryanodine receptor (SR Ca²⁺ release channel) and these effects may interact in complex ways (Clancy & Rudy, 2002; Grandi *et al.* 2007; Wagner *et al.* 2009, 2011; Maier, 2011; Ma *et al.* 2012; Horvath *et al.* 2013; D. M. Zhang *et al.* 2014). CaMKII can also modulate the expression of certain K⁺ channel subunits (Wagner *et al.* 2009). The composite effect of CaMKII upregulation is pro-arrhythmic (Wagner *et al.* 2006, 2009; Grandi *et al.* 2007; Hund *et al.* 2008).

CaMKII was also reported to interact with β IV-spectrin, and that interaction may position CaMKII to phosphorylate the channel to alter I_{NaL} gating (Hund *et al.* 2010). Three different sites in the intracellular loop linking domains I and II have been shown to be phosphorylated by CaMKII (S516, S571 and T594) and are implicated in the CaMKII-induced changes in I_{Na} gating (Hund

et al. 2010; Ashpole *et al.* 2012). Indeed, phosphomimetic mutations at these three sites could mimic some of the effects and non-phosphorylatable mutants could block CaMKII effects on Na⁺ channel availability, intermediate inactivation and I_{NaL} . However, the relative roles of these three sites are somewhat controversial, and more than one site may be involved. Clarifying the molecular mechanism linking specific site phosphorylation and functional effects on I_{Na} gating remains an important area of study.

Question or controversy: CaMKII regulation of late Na⁺ current

The specific functional roles of the three CaMKII phosphorylation sites (S516, S571 and T594) on Na_v1.5 are subjects of debate.

What are the detailed mechanisms of CaMKII activation of I_{NaL} ?

The details of the bi-directional feedback between I_{NaL} and CaMKII still need to be resolved.

The role of CaMKII in activating I_{NaL} is compelling, even though the molecular details await further clarification. Recent studies have continued to show that CaMKII inhibition reduces I_{NaL} measured using the ^{self}AP-clamp recording (Horvath *et al.* 2013). Maltsev and coauthors have also shown that effects of CaMKII inhibition on I_{NaL} are stronger in ventricular myocytes isolated from dogs with heart failure vs. normal dogs (Maltsev *et al.* 2008b). The reverse is also true that an increased late I_{Na} can also activate CaMKII (Yao *et al.* 2011), setting up a deleterious positive feedback cycle between I_{Na} and CaMKII. That is, greater Na⁺ entry via I_{NaL} increases [Ca²⁺]_i (via Na⁺/Ca²⁺ exchange) and that promotes greater CaMKII activation and further increase in I_{NaL} (Morotti *et al.* 2014). Indeed, some CaMKII effects have been integrated into detailed computational models to better understand crosstalk and feedback (Veldkamp *et al.* 2000; Erickson & Anderson, 2008; Hund *et al.* 2008; Saucerman & Bers, 2008; Wagner *et al.* 2009, 2011; Moreno *et al.* 2013; Morotti *et al.* 2014). Model predictions may serve as a guide to future experiments in order to gain a more complete understanding of the complex Na⁺ and Ca²⁺ regulation systems.

Phosphorylation of Na_v1.5 channels by CaMKII is a potential arrhythmogenic mechanism in heart failure and has emerged as a critical area of investigations in cardiac electrophysiology (Grandi *et al.* 2007; Herren *et al.* 2013). However, direct identification of key phosphorylation sites in native cardiac Na⁺ channel remains unclear to date. Understanding the CaMKII phosphorylation of Na⁺ channels will be critically important to the ongoing efforts to develop new therapeutic strategies using inhibitors of CaMKII and I_{NaL} .

Distinct population of Na⁺ channels in cardiac myocytes.

An important aspect of Na⁺ channel regulation is the channel spatial distribution. Two separate pools have been identified, one at intercalated discs and the other at the lateral and the T-tubular membrane. Those at the lateral membrane interact with dystrophin and syntrophin proteins, while the membrane-associated guanylate kinase protein SAP97 may organize channels at intercalated discs. The existence of these two pools immediately raises interesting physiological and pathophysiological questions (Shy *et al.* 2013). For example, based on work from Hund and colleagues regarding CaMKII targeting to intercalated discs through its β IV-spectrin interaction, the SAP97 pool of Na⁺ channels at the intercalated discs might contribute more to I_{NaL} than the lateral dystrophin/syntrophin pool. More recent evidence for the multiple Na_V1.5-pool model has been published by Shy *et al.* (2014), using genetically modified mice where the SAP97 and syntrophin interaction domain was truncated (at the last 3 amino acids of Na_V1.5). That caused an exclusive down-regulation of Na_V1.5 at the lateral sarcolemma (Fig. 2) leading to slow impulse propagation and increased anisotropy of conduction (Shy *et al.* 2014).

Question or controversy: localization of Na⁺ channels in cardiac myocytes

How does Na_V1.5 localization affect function, regulation and stabilization?

What are the interacting proteins in complex with Na_V1.5 and how do they modify function?

These recent results are challenging the role of SAP97 in targeting and stabilizing Na_V1.5 at the intercalated discs, and suggest that this interaction may have other roles that remain to be determined. What happens to these pools in

heart failure and the consequent increase in I_{NaL} remains an important question to be addressed.

Na⁺ channel structure

In the session on 'Na⁺ channel structure and function', William Catterall talked about structural studies and Vladimir Yarov-Yarovoy presented modelling studies. Richard Aldrich led the discussion with the panelists Mark Cannell, Walter Chazin, Eric Sobie and Jon Sack.

Understanding the structures adopted by Na_V channels can profoundly advance therapeutic strategies for treating cardiac disorders if cardiac Na_V channel subtypes could be selectively modulated. There are nine human Na_V channel subtypes that are expressed in neuronal, cardiac and muscle cells (Catterall *et al.* 2005). The Na_V1.5 is the primary Na_V channel isoform expressed in cardiac muscle (Sheldon *et al.* 1986; Rogart *et al.* 1989; Gellens *et al.* 1992). Additionally, significant levels of expression of neuronal Na_V1.1, Na_V1.2, Na_V1.3, Na_V1.4 and Na_V1.6 channels have also been detected in cardiomyocytes (Maier *et al.* 2004; Kaufmann *et al.* 2013; Westenbroek *et al.* 2013). To selectively target and modulate the function of cardiac Na_V channels, ligands are needed that distinguish between different Na_V channel subtypes. The key to the rational design of subtype-selective drugs is to advance our understanding of structure and function of cardiac Na_V channels at atomic resolution.

Structure of Na_V channels. Recently, the first structures of a bacterial Na_V channel were solved at high resolution (Payandeh *et al.* 2011, 2012; McCusker *et al.* 2012; Zhang *et al.* 2012; Shaya *et al.* 2014). These structures provide useful templates for structural modelling of human Na_V channels (Fig. 3). Prokaryotic Na_V channels

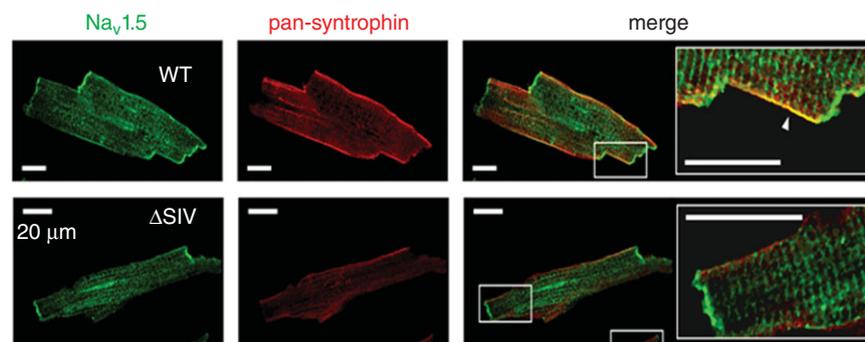


Figure 2. Na_V1.5 localization

Upper panels show confocal images of mouse ventricular myocyte with dual immunofluorescence staining. Na_V1.5 (green) is expressed at the intercalated discs, T-tubules and lateral membrane. Punctate staining is most-likely at T-tubules. Syntrophin is only expressed at the lateral membrane where it co-localizes with Na_V1.5 (arrow in merge showing the yellow region of co-localization). Lower panels show myocytes from genetically modified mice (truncation of the last three residues of Na_V1.5 interacting with syntrophins and SAP97, Δ SIV) illustrating the reduction of Na_V1.5 expression exclusively at the lateral membrane (from Shy *et al.* 2014, with permission).

are formed by four identical subunits that form two- or fourfold symmetric structures (Payandeh *et al.* 2011, 2012; McCusker *et al.* 2012; Zhang *et al.* 2012; Shaya *et al.* 2014). Eukaryotic Na_V channels are different in that the pore is formed from a single copy of a protein composed of four homologous domains (I–IV). Importantly, each of the domains is similar to a prokaryotic subunit. In all Na_V channels, each domain contains six transmembrane segments (S1–S6), with segments S1–S4 forming the voltage-sensing domain (VSD) and segments S5 and S6 forming the pore-forming domain (PD) (Fig. 3) (Payandeh *et al.* 2011; Catterall, 2014). The loop between segments S5 and S6 forms the narrow selectivity filter that is stabilized by P1 and P2 helices (Fig. 3). The S4 segment in the VSD has several gating-charge-carrying arginines that play a key role in the mechanism of voltage sensing. By grafting extensive experimental data of decades of functional and biochemical studies on eukaryotic Na_V channels onto the structure of bacterial Na_V channels, atomistic details of mammalian Na_V channel structure–function relationships are beginning to emerge.

What can bacterial Na_V channels tell us? Breakthroughs in membrane protein structure are occurring at a startling pace, yet atomic resolution electron densities of mammalian Na_V channels are unlikely to emerge in the next few years. Despite the heroic efforts of

many laboratories, it remains a great challenge to prepare large quantities of high quality, functional eukaryotic Na_V channel proteins. Hence, we are reliant on bacterial Na_V channels for atomistic structures. While sequence conservation within each mammalian Na_V domain is remarkably high across Eukarya, each domain contains distinct sequence differences that are crucial for voltage-dependent activation and inactivation (Chanda & Bezanilla, 2002; Chanda *et al.* 2004; Campos *et al.* 2007, 2008; Capes *et al.* 2013; Catterall, 2014). Detailed studies showing different contributions of the four domains of mammalian Na_V channels make it clear that not all questions about channel gating can be answered from the single domain bacterial Na_V channels (Chanda & Bezanilla, 2002; Chanda *et al.* 2004; Campos *et al.* 2007, 2008; Capes *et al.* 2013). While the selectivity filter of bacterial Na_V channels is formed by an ‘EEEE’ motif, the selectivity filter of eukaryotic Na_V channels is formed by the ‘DEKA’ motif. Clearly, however, the bacterial Na_V channels share Na⁺ selectivity, gating and pharmacological features that are very similar to mammalian Na_V channels. Most significantly, recent studies from the Catterall lab suggest the possibility that slow inactivation of Na⁺ channels, which is most crucial for determining I_{NaL} during the AP, may result from asymmetric closure of the intracellular pore mouth (Payandeh *et al.* 2012). This has significant implications for the effects of open channel and other blockers with complex use-dependent effects. It remains to be seen whether the mechanisms underlying these properties are related or convergent at the atomic level.

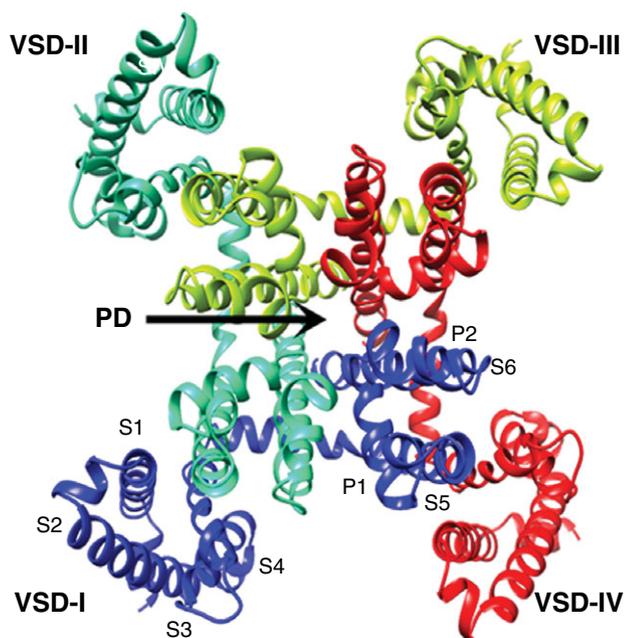


Figure 3. Structure of a sodium channel

Voltage-sensing domains (VSD-I, VSD-II, VSD-III, and VSD-IV) and pore-forming domain (PD) are labelled. Transmembrane segments S1–S6, and selectivity filter region pore helices P1 and P2 are labelled for domain I (blue). Each domain is a different colour.

Antiarrhythmic and local anaesthetic drug binding site is conserved among Na_V channel subtypes. Ion conduction through Na_V channels can be directly altered by the interaction of antiarrhythmic and local anaesthetics such as lidocaine (lignocaine) to a receptor site formed within the PD inner cavity (Ragsdale *et al.* 1994; Yarov-Yarovoy *et al.* 2001, 2002). However, because residues forming the antiarrhythmic and local anaesthetic drug receptor site in the PD are highly conserved among all Na_V channels, all currently available therapeutic drugs targeting Na_V channels have only modest subtype selectivity.

Structure-based drug design: a way forward for Na_V therapeutics. The variable functional regions of Na_V channels are prime targets of natural toxins from venoms of predatory creatures such as scorpions, spiders, snakes and cone snails (Keizer *et al.* 2003; Catterall *et al.* 2007; Zhang *et al.* 2007; Bosmans & Swartz, 2010). These toxins serve as useful molecular tools to study human Na_V channel structure and for design of novel modulators of Na_V channel function. Future studies will focus on targeting unique amino acid residues within three distinct

regions in human Na_v channel structure: the extracellular side of the selectivity filter in the pore-forming domain (Walker *et al.* 2012), the extracellular side of the voltage-sensing domain II that is responsible for channel activation and is a target of a number of peptide toxins (Chanda & Bezanilla, 2002; Campos *et al.* 2007; Catterall *et al.* 2007; Bosmans & Swartz, 2010), and the extracellular side of the voltage-sensing domain IV that is responsible for channel inactivation and is a target of a number of peptide toxins and small ligands (Chanda & Bezanilla, 2002; Catterall *et al.* 2007; Campos *et al.* 2008; Capes *et al.* 2013; McCormack *et al.* 2013). An advantage of targeting the Na_v channel voltage-sensing domains is that instead of simply impeding the Na⁺ conduction through the pore, these allosteric modulators may fine-tune human Na_v channel activity in a specific manner. High resolution structural templates from the expanding database of bacterial Na_v channel X-ray structures and voltage-sensing domain X-ray structures of homologous ion channels (Payandeh *et al.* 2011, 2012; McCusker *et al.* 2012; Zhang *et al.* 2012; Shaya *et al.* 2014), in combination with experimental data-driven computational structural modelling (Henrion *et al.* 2012; Jensen *et al.* 2012; Vargas *et al.* 2012; Yarov-Yarovoy *et al.* 2012) will aid in probing human Na_v channel structure and attempts at rational design of novel high specificity modulators of Na_v channel function.

Question or challenge: Na⁺ channel structure

Atomic resolution of the structure of human Na⁺ channels will allow for isoform-specific pharmacological targeting.

Major challenges remain to produce large quantities of pure mammalian Na_v proteins.

How to model equilibrium, transition and unstable states using structural tools?

Retrograde trafficking is an emerging field with many unanswered questions.

What are the limitations of atomistic structural models of Na_v channels? The structural approaches to drug design require the Na_v channel conformations captured to be similar to those adopted by Na_v channels in the cell membrane. However, while the structures observed are necessarily at a stable equilibrium, Na_v channel function is a dynamic process. In addition to conformations occupied at equilibrium, the unstable intermediates and transition states that dictate the kinetics of Na_v channel opening are of the utmost importance. To understand these structurally is even more of a challenge. X-ray structure-determination methods can capture meta-stable closed, open or inactivated Na_v channel states, but to atomistically understand kinetics, structural information

on the transiently populated Na_v channel states is also required. What does a Na_v channel's structure look like as it vaults over an activation barrier? Experimental techniques are unlikely to directly capture transient Na_v channel states. However, computational structural modelling provides a means to address these critical issues. For example, understanding Na_v channel kinetics requires characterizing the range of conformational states populated and knowing what the energy of the transient states are compared to all of the other states. Computational simulation such as molecular dynamics appears to be our best hope to understand these transitions. Empirical validation of simulation-generated hypotheses will be critical to evaluating the effectiveness of this approach. With sufficient structural understanding, careful modelling of functional coupling and robust experimental validation this may be achievable. In that event, computational methods may be capable of designing drugs to stabilize specific Na_v channel conformations.

In recent years methods for ligand design, exemplified by the Rosetta approach, have proven capable of designing high affinity peptide ligands *de novo*, for a variety of protein targets. These computational chemistry approaches will doubtless advance in coming years and should provide a way forward for structure-based drug design, assuming atomic coordinates of Na_v channel conformations to target can be modelled. Thus, the burning question is, do we have structural models that are sufficient in their atomistic detail to understand the channel gating mechanisms? An exciting new direct electron detection technology for electron microscopy (Cao *et al.* 2013; Liao *et al.* 2013) appears to be extremely promising for directly determining high-resolution structures of mammalian Na_v channels and should this prove successful, these will probably provide access to multiple states. However, until eukaryotic structures are captured, understanding how bacterial Na_v channels work at the atomic level will serve to provide the primary basis for studies of structure and function of mammalian Na_v channels.

Trafficking, sequestration and complexing

In the session on 'Trafficking, sequestration and complexing', Peter Mohler presented work on Na⁺ channel sequestration and Isabelle Deschenes presented work on Na⁺ channel trafficking. Robin Shaw led the discussion with Nipavan Chiamvimonvat.

A well-documented but not widely appreciated aspect of ion channel biology, including cardiac ion channel biology, is that individual ion channels have half-lives in the order of hours. Connexin43 (Cx43) gap junction protein has a half-life of about 1–3 h (Beardslee *et al.* 1998; Smyth

et al. 2014). Potassium, calcium and sodium–calcium exchanger current channels have half-lives that are reported to be in the range of 2–8 h (Chien *et al.* 1995; Egger *et al.* 2005; Colley *et al.* 2007; Di Biase *et al.* 2011). It is not clear whether channel half-lives on the plasma membrane are longer or shorter than the overall protein half-life, or whether post-translational modifications (e.g. phosphorylation) can influence this. Indeed, it has been reported that different pools of channels exist within the cardiomyocyte (Petitprez *et al.* 2011). In cardiac myocytes, channels such as the Na⁺ channel may have a slightly extended half-life of about 35 h (Maltsev *et al.* 2008aa); however, the half-lives of channels are likely to be different based on cell type and specific membrane partners. The implications for a turnover on the scale of several to tens of hours is that, in the course of any given day, the bulk of ion channels in an individual cardiomyocyte are regenerated and newly positioned. Thus the formation and *trafficking* of channels is considered as a dynamic ongoing process, and expected to have a critical impact on overall channel function.

Ion channel trafficking can be categorized into the three groups: *forward trafficking*, which is the transport of *de novo* post-translation channels and recycled channels from the cytoplasm to the membrane; *membrane behaviour*, which is the behaviour of channels once trafficked to membrane and their lateral movement to other local and distant regions of membrane; and *retrograde trafficking* or internalization and transport to degradation or recycling pathways.

Forward trafficking. Regarding forward trafficking, many components of the traditional cytoskeleton are responsible for channel delivery. Research from Shaw and colleagues has explored targeted delivery for cardiac ion channels, which is based on the concept that ion channels exit the endoplasmic reticulum (ER)/Golgi apparatus and are delivered directly to specific subdomains of the plasma membrane. Channels are transported along microtubule highways and specificity of targeted delivery occurs as a result of a combination of microtubule plus-end tracking proteins, the channel itself, and a membrane anchor (Shaw *et al.* 2007; Hong *et al.* 2010; Smyth *et al.* 2010). Under the conditions of oxidative stress that occur in ischaemic heart disease and heart failure, microtubule plus-end tracking proteins are displaced and forward delivery of channels impaired (Smyth *et al.* 2010). Absence of anchor proteins can also affect forward trafficking. Loss of the membrane anchor BIN1 diminishes forward trafficking of the L-type calcium channel in heart failure (Hong *et al.* 2012). The actin cytoskeleton is also involved in channel forward trafficking (Smyth *et al.* 2012; S. S. Zhang *et al.* 2014). Indeed, the roles of cytoskeletal proteins in channel trafficking may represent a unified theme for other types of

ion channels. For example, both α -actinin2 and filamin A are critical for K⁺ channel trafficking as reported by the Chiamvimonvat lab (Lu *et al.* 2009; Rafizadeh *et al.* 2014).

Question or challenge: Na⁺ channel trafficking

What are the mechanisms of forward trafficking? How do ion channels regulate trafficking?

Major challenges remain due to the lack of high quality antibodies for cardiac ion channels, transporters and associated membrane proteins.

The mechanisms of ion channel forward trafficking are only beginning to be determined and include novel mechanisms such as the role of ankyrin adaptor proteins on channel localization, with ankyrin-G binding to and regulating Na_v1.5 localization (Lowe *et al.* 2008; Dun *et al.* 2013) and ankyrin-B regulating the membrane targeting and subsequent regulation of the Na⁺/Ca²⁺ exchanger, Na⁺/K⁺-ATPase, Ca_v1.3 and *myo*-inositol 1,4,5-trisphosphate (IP₃) receptor (Cunha & Mohler, 2011). Notably, defects in ankyrin-based sequestration pathways have been tightly linked with arrhythmia phenotypes in animal models as well as humans. For example, mutations in Na_v1.5 that lack the ability to bind ankyrin-G are now known to cause aberrant Na_v1.5 trafficking and result in human Brugada syndrome (Mohler *et al.* 2004). Furthermore, ankyrin-B dysfunction is linked with sinus node dysfunction, atrial fibrillation, conduction defects, and potentially fatal ventricular arrhythmia (Mohler *et al.* 2003; Le Scouarnec *et al.* 2008; Cunha *et al.* 2011). Fibroblast homologous growth factors have also been shown to be potent regulators of Na_v1.5 and Ca_v1.2 localization to the sarcolemma (Wang *et al.* 2011; Hennessey *et al.* 2013).

Ion channels can also autoregulate forward movement. For example, coexpression of mutant and wild-type (WT) Na⁺ channel subunits has been shown to reduce Na⁺ current. Surprisingly, the reduction was not because of altered gating but reduced membrane expression. It appears that the trafficking signal involves a cluster of α subunits and that the signal is aberrant in mutant/WT clusters. Furthermore, it was recently found that the Cx43 mRNA transcript can undergo alternative translation, utilizing internal in-frame AUG start sites with the production of as many as six significantly smaller C-terminal truncation isoforms (Smyth & Shaw, 2013). At least one of the smaller isoforms (GJA –20 kDa, which is a nomenclature derived from gene name and the size of the alternative translated isoform) assists with full-length Cx43 exit from the ER/Golgi. It is likely that other cardiac ion channels undergo alternative translation as well, explaining both the origin of smaller fragments of unclear origin as well as potentially non-ion-flux-related cellular functions of channel proteins.

As limitations of the analysis of forward trafficking, technical challenges in protein identification and cellular imaging were discussed. A major advance is the use of fluorescent proteins that fuse with the channel proteins of interest to follow the trafficking of channel proteins and relationship to cytoskeleton components and membrane anchors in real-time. More recently, polydimethylsiloxane (PDMS)-based patterning has been employed to force cell lines and neonatal cardiomyocytes to grow into predefined cell-pair regions allowing for reproducible cultured cell geometry to better control and quantify the cytoskeleton dynamics and behaviour of channel movement to cell–cell borders. While exciting new imaging techniques are being developed, future work must address fundamental issues with the lack of high quality antibodies for cardiac ion channels, transporters and associated membrane proteins.

Membrane behaviour. Early understanding of the plasma membrane lipid bilayer was that it is a relatively non-constricted surface, and channels and other membrane proteins could diffuse freely in the membrane as well as anchor to scaffolding proteins. This fluid mosaic model is increasingly appreciated as being more mosaic than fluid (Nicolson, 2014). Many transmembrane proteins such as vesicular stomatitis virus glycoproteins freely diffuse, but some ion channels may not enjoy much mobility. For Cx43 channels as well as other cardiac ion channels, the recent evidence has focused on local rearrangements in their respective membrane sub-domains. The cardiac intercalated disc is now viewed as consisting of at least two pools of channels, those within a gap junction plaque, and those in the immediate vicinity in a region known as the perinexus (Rhett & Gourdie, 2012). Analyses of models relating to arrhythmogenic right ventricular cardiomyopathy are revealing channel–channel interactions once they are in the plasma membrane. The Isom group has explored the role of the Na⁺ channel β subunit which can regulate cell–cell adhesion (Patino & Isom, 2010). The Delmar group has reported evidence that Cx43 channels at the intercalated disc help with Na_v1.5 channel localization to this region (Jansen *et al.* 2012).

Retrograde trafficking. Retrograde trafficking is also a highly regulated yet poorly understood aspect of cardiac ion channel biology. The actin cytoskeleton is the most prominent member of the cytoskeleton implicated, and different myosin motors may also be involved in forward *versus* retrograde actin trafficking (Schumacher-Bass *et al.* 2014).

Post-translation modification, specifically alteration of phosphorylation status, is associated with an increase in channel internalization. Dephosphorylation of the casein kinase-related serines of the Cx43 C-terminus, as occurs

during stress, reduces the channel density of Cx43 and increases the likelihood of ventricular arrhythmogenesis (Remo & Fishman, 2010). The dephosphorylation of the casein kinase-related serines probably accounts for the dephosphorylation-related band shifts noted for Cx43 in ischaemia and ischaemia–reperfusion injury (Smyth *et al.* 2014). Like all cardiac ion channels, the Cx43 C-terminus has multiple regions subject to altered phosphorylation. It was recently found that the ubiquitous scaffolding protein 14-3-3 is necessary for initiating a well-sequenced cascade of phosphorylation and dephosphorylation, leading to channel ubiquitination and internalization (Smyth *et al.* 2014). Multiple post-translation modification steps of other ion channels are also likely to occur independently of each other, but in a specifically sequenced cascade, regulating the channel survival time once at the plasma membrane.

The emerging picture is one in which Na⁺ channels are specifically targeted for forward trafficking to the intercalated disc or the lateral membranes. Channels in different environments have different molecular partners that differentially alter channel characteristics. I_{NaL} , which plays an important role in arrhythmogenesis, might arise not from a different channel isoform but from increased expression in a different location, namely the intercalated disc.

The interaction of Na⁺ channel α subunits is another emerging area of research. Coexpression of mutant and WT α subunits reduces Na⁺ current not by altered gating as one might expect but by reduced membrane expression. Whether this is due to reduced forward trafficking or enhanced sequestration is unclear. Thus a Na⁺ channel should not be viewed as a lone voice but rather as part of a chorus that moves (or not) as a unit. The dynamism of channel turnover – potentially as much as 2 or 3 times per day – could account for changes in channel expression in disease. This opens the exciting possibility for diurnal changes in channel expression in quantity or location. Such changes would result in altered Na⁺ current characteristics that might contribute to the diurnal fluctuations in arrhythmia frequency.

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Additional information

Competing interests

None declared.

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