Targeting ion channels for the treatment of gastrointestinal motility disorders

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Abstract: Gastrointestinal (GI) functional and motility disorders are highly prevalent and responsible for long-term morbidity and sometimes mortality in the affected patients. It is estimated that one in three persons has a GI functional or motility disorder. However, diagnosis and treatment of these widespread conditions remains challenging. This partly stems from the multisystem pathophysiology, including processing abnormalities in the central and peripheral (enteric) nervous systems and motor dysfunction in the GI wall. Interstitial cells of Cajal (ICCs) are central to the generation and propagation of the cyclical electrical activity and smooth muscle cells (SMCs) are responsible for electromechanical coupling. In these and other excitable cells voltage-sensitive ion channels (VSICs) are the main molecular units that generate and regulate electrical activity. Thus, VSICs are potential targets for intervention in GI motility disorders. Research in this area has flourished with advances in the experimental methods in molecular and structural biology and electrophysiology. However, our understanding of the molecular mechanisms responsible for the complex and variable electrical behavior of ICCs and SMCs remains incomplete. In this review, we focus on the slow waves and action potentials in ICCs and SMCs. We describe the constituent VSICs, which include voltage-gated sodium (Na_V), calcium (Ca_V), potassium (K_V, K_{Ca}), chloride (Cl^-) and nonselective ion channels (transient receptor potentials [TRPs]). VSICs have significant structural homology and common functional mechanisms. We outline the approaches and limitations and provide examples of targeting VSICs at the pores, voltage sensors and alternatively spliced sites. Rational drug design can come from an integrated view of the structure and mechanisms of gating and activation by voltage or mechanical stress.

Keywords: functional GI, GI motility, ion channel, voltage gated ion channel, potassium voltage gated ion channel, sodium voltage gated ion channel, calcium voltage gated ion channel, chloride channel

Gastrointestinal functional and motility disorders

Gastrointestinal (GI) functional and motility disorders are common and commonly morbid. In the broadest sense, GI functional and motility disorders refer to a group of disorders whose symptomatology is related to motor, sensory or even secretory function of the GI tract. The term 'functional' refers to the subgroup of disorders that do not yet have a well-defined pathophysiology. This broad definition means that the symptoms are nonspecific and include nausea, vomiting, bloating, abdominal discomfort or pain, constipation or diarrhea. Some of the most common motility disorders encountered in practice are achalasia, gastroparesis, intestinal pseudo-obstruction and slow transit constipation. Common functional GI disorders such as functional dyspepsia and irritable bowel syndrome (IBS) are highly prevalent in the community. For example, IBS has an estimated prevalence of 15–22% in Western society and by itself accounts for 3% of all primary care visits and up to 60% of the referrals for secondary care [Ford *et al.* 2008]. GI motility disorders are also not standalone pathologies; they may be

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complications of other systemic illnesses, such as diabetes, which can result in diabetic gastroparesis in a subset of patients [Camilleri *et al.* 2011; Kashyap and Farrugia, 2010]. Finally, intestinal pseudo-obstruction and other less common GI motility disorders are associated with substantial mortality risk.

Pathophysiology

GI functional and motility disorders have a multifactorial pathophysiology. Pathologies responsible for these disorders span the central (CNS) and peripheral (enteric) (ENS) nervous systems, interstitial cells of Cajal (ICCs), smooth muscle cells (SMCs), and immune cells. For example, IBS involves a complex interplay between multiple potential pathologic factors, including abnormal pain signaling due to both central perception and peripheral sensitivity, infectious or postinfectious causes and disordered motility of the GI tract [Ford and Talley, 2011]. While the complex multivariate nature of these disorders is responsible for many diagnostic and therapeutic challenges [Ford and Talley, 2011], a particular advantage is that multisystem pathophysiology provides a rich source of potential targets. We defer the extensive discussion on targeting ion channels in the CNS and ENS to other excellent reviews [Gourine *et al.* 2009; Storr and Sharkey, 2007; Galligan, 2004, 2002; Cervero and Laird, 2003; Smith *et al.* 2003; Galligan and North, 1988]. Instead, we focus on the effectors of the GI tract, the motor cells: SMCs and ICCs. In the GI tract, the ICCs have several functions [Sarna, 2008] including generating and propagating electrical activity [Thomsen *et al.* 1998; Huizinga *et al.* 1995], setting SMC membrane potential [Farrugia *et al.* 2003], mediating neuronal input [Powley *et al.* 2008], and as mechanosensors [Won *et al.* 2005; Strege *et al.* 2003b]. The ICC and SMC system coordinates electromechanical coupling [Der-Silaphet *et al.* 1998] and mechanoelectrical feedback [Kraichely and Farrugia, 2007]. There are at least three distinct advantages of targeting the effector cells. First, SMCs and ICCs are essential for normal motility, so dysfunction in these cell types is most likely pathogenic [Farrugia, 2008]. Second, the final effector targets allow direct intervention, limiting side effects that hamper approaches involving upstream targets. Third, drug delivery to these cells may be facilitated by their location close to the gut lumen.

Electromechanical functions

GI tract wall organization underlies its electromechanical functions. Both cyclical and stimulated contractions of the GI tract require electrical excitation and excitation–contraction coupling. GI motility is the result of coordinated activity of extrinsic nerves, the ENS, immune cells, ICCs and SMCs. Yet, the GI tract is able to function independently of external neuronal input. We know that ICCs are fundamental for the generation and propagation of the electrical cyclical activity in the GI tract [Thomsen *et al.* 1998]. In the small intestine, ICCs around the myenteric (Auerbach's) plexus (ICC-MYs) between the circular and longitudinal muscle layers are responsible for the generation the cyclical activity, known as slow waves [Kito *et al.* 2005]. In the colon submuscular ICCs (ICC-SMs) appear to be required for slow wave generation [Lee *et al.* 2009]. Slow waves are the cyclical electrical events that depolarize the ICCs for seconds from its resting membrane potential to a more positive voltage, but the resting membrane potential, amount of depolarization and frequency of the slow waves are variable through the GI tract [Hara *et al.* 1986]. Other ICC networks, such as intramuscular ICCs (ICC-IMs) propagate and amplify slow waves and thus regulate motility [Cho and Daniel, 2005] (Figure 1A). The slow waves are then transmitted to the SMCs, which also depolarize cyclically. The result of the SMC depolarization is activation of calcium entry and contraction, also known as excitation–contraction coupling. In the following sections we provide some details on the slow wave generation in the ICCs and propagation to the SMCs with particular focus on the involved voltage-sensitive ion channels (VSICs) and their potential as drug targets.

Electrical activity can be studied at increasingly reductionist levels. A major objective in the study of ion channels of SMCs and ICCs is to identify the molecular identities and biophysical properties. A general multidisciplinary approach is a combination of electrophysiology, pharmacology and molecular biology. Ion channels can be classified by their ion selectivity (e.g. K^+, Na^+, Ca^{++}, Cl^-, nonselective cation), mode of activation (voltage, ligand, mechanical), and time-dependent properties of ion conduction (kinetics). Ion substitution and ion channel modulators can be used as early screening approaches for the presence of particular ion channel species [Barajas-Lopez *et al.* 1989]. However, the
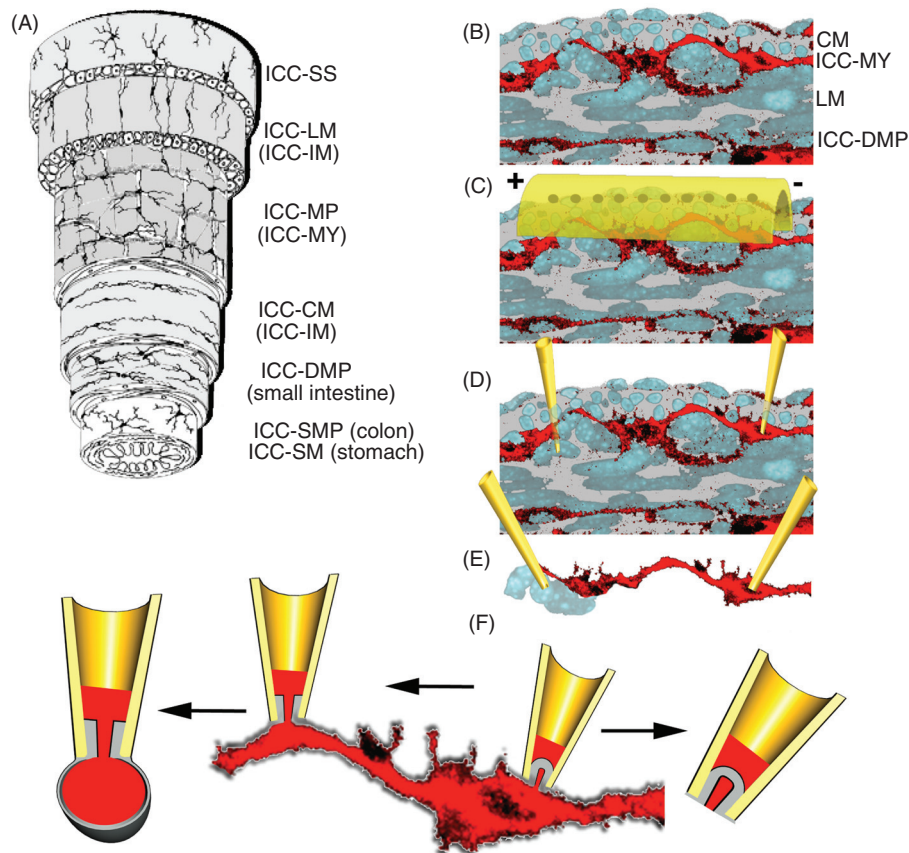
Figure 1. Reductionist electrophysiology approaches for the study of ion channel activity in the gastrointestinal (GI) tract. (A) Diagram of the GI wall structure (reproduced with permission from [Hanani *et al.* 2005]) showing interstitial cells of Cajal (ICC) networks interspersed between the muscle layers and submucosa. (B) Cross section of a segment of murine small bowel with nuclei of the smooth muscle cells (SMCs) in gray and ICCs in red (circular muscle layer on top, mucosa on bottom). (C) Overlying the tissue is a set of extracellular electrodes. (D) Dissected segments may be impaled by intracellular electrodes so that single cell electrical activity is recorded, whether from ICCs (right electrode), SMCs (left electrode) or both. (E) Tissue may be further dissected into isolated small strands of muscle/ICCs. (F) Completely dissociated tissue or cells that heterologously express channels of interest may be voltage clamped in multiple configurations (from left to right: outside out, whole cell, cell attached and inside out).

detailed study of the electrophysiological operation requires experimental approaches that span from the tissue level down to the molecular level. Extracellular recording electrodes can be used to record electrical activity at the tissue and organ levels [Egbuji *et al.* 2010] (Figure 1B) and tools are being developed for endoscopic use [Coleski and Hasler, 2004]. However, the study of the mechanisms that underlie electrical function at the organ level also requires examination on smaller scales. To this end, muscle strips may be obtained from animals and human surgical specimens. The strips may be dissected and can then be impaled using sharp microelectrodes (Figure 1C). The recorded electrical behavior is a composite of activities of ion channels of

different families and from many surrounding cells [Hara *et al.* 1986]. These strips can be further dissociated into small bundles that allow closer inspection of the rhythmic behaviors, and often with an ability to control electrical parameters such as voltage (voltage clamp) [Beckett *et al.* 2004] (Figure 1D). Complete dissociation of the tissue allows detailed examination of single cells. The cells of interest can be identified visually or by specific molecular markers, such as Kit [Rich *et al.* 2002; Thomsen *et al.* 1998]. Individual cells can be sampled using electrophysiology in whole cell [Hamill *et al.* 1981] or single channel patch clamp modes [Neher and Sakmann, 1976] (Figure 1F). Finally, once the ion channels of interest are identified and

cloned, these can be heterologously expressed in cell lines and fine details of channel operation can be studied [Beyder *et al.* 2010]. Single cell and patch approaches provide an experimental template for examination of potential pharmaceutical interventions, but these are notoriously low throughput. Recently, multipatch platforms have been developed to extend cellular electrophysiology studies into the high-throughput realm [Estacion *et al.* 2010].

Basic voltage-gated ion channel structure

Voltage-gated ion channels gate predominantly in response to voltage. Other channels have some varying sensitivity for voltage, but their gating is dominated by other stimuli. We define both as VSICs and make them the focus of this review. The reasons for this broader channel selection are that VSICs have central roles in the physiology of GI motility and that they are structurally and functionally homologous. The basic building block of a VSIC is characterized by a single domain of a typical K_V channel shown in Figure 2A (top). Functional potassium (K_V), big K (BK) and transient receptor potentials (TRPs) are tetramers, while sodium (Na_V) and calcium (Ca_V) channels are monomers with four homologous domains (Figure 2A, middle). The S1–S4 from each domain serve as the voltage sensors, and the charge on S4 determines the amount of voltage sensitivity (high for K_V, Na_V, Ca_V, low for TRP, for example). The S5 and S6 from the four domains fold to form the pore (blue), with the S5–S6 linker (green) serving as a selectivity filter. The molecular architecture of these channels is emerging as recent crystal structures are showing a cruciform layout with the voltage sensors making an extensive interaction with the surrounding lipids (Figure 2B). The newly discovered calcium-activated-chloride channel (Ano1) has a topology that is different from the traditional VSICs, specifically that the voltage sensor is on the intracellular loop, but little is otherwise known about this channel's structure (Figure 2A, bottom). Multiple potential drug targets for VSICs are presented as a diagram in Figure 2C and are explained below.

Pacemaker ICCs generate and propagate slow waves to SMCs which produce contractions. The mechanisms of cyclical electrical activity in the GI tract have been studied extensively (Figure 3A). Nevertheless, the ion channel that gives rise to pacemaker current which initiates the slow wave is still not established. The main candidates are a calcium-inhibited nonselective cation channel [Koh *et al.* 2002], a calcium-activated chloride channel [Gomez-Pinilla *et al.* 2009; Zhu *et al.* 2009] or a combination of both [Namkung *et al.* 2011; Kito and Suzuki, 2003]. Pacemaker current depolarization turns on the local T-type Ca^{++} channels [Lee *et al.* 2007] (Figure 3E), and in humans and dogs also $Na_V1.5$ [Strege *et al.* 2003a] (Figure 3D) producing further depolarization and the upstroke of the slow wave. The slow wave plateau and the repolarization phase are likely a combination of multiple channels. These include Ano1, which after its involvement in the upstroke should pass outward currents, repolarizing the membrane at potentials more positive than –50 mV [Zhu *et al.* 2009] (Figure 3C). K channels, including ERG [McKay *et al.* 2006], delayed rectifiers [Huizinga *et al.* 2004; Hatton *et al.* 2001], BK and Ca^{++} activated K channels also contribute to repolarization [Zhu and Huizinga, 2008] (Figure 3F). The resting membrane potential is maintained by a combination of the sodium, potassium and NS cation channels. ICCs also express L-type Ca channels whose function is still not established. Slow waves generated by the ICCs are passively conducted to the SMCs, SMC depolarization provides the stimulus for opening of the voltage-dependent L-type calcium channels, leads to a Ca^{++} mediated action potential that consists of a plasmalemmal Ca^{++} influx, and a further Ca^{++} release from the internal stores, so-called calcium-activated calcium release. Increase in the cytoplasmic calcium is the impetus for excitation–contraction coupling.

Voltage-sensitive chloride selective channels

Voltage-sensitive Cl^- channels have been described in the GI tract in the SMCs [Sun *et al.* 1992] and ICCs [Huizinga *et al.* 2002], with Ca^{++}-activated Cl^- current (CaCC) in various smooth muscle systems (as reviewed by Large and Wang [1996]). CaCC conductance was suggested as essential in slow wave generation, since a block of Cl^- conductance resulted in the loss of slow wave, but the multiple molecular candidates did not pan out [Ferrera *et al.* 2010]. A recent determination that the CaCC anoctamin 1 (Ano1) encoded by TMEM16A [Caputo *et al.* 2008] in the muscularis propria is relatively specific to ICCs in mice and humans [Gomez-Pinilla *et al.* 2009]. ANO1 is vital for slow wave function in ICCs [Zhu *et al.* 2009], as mice lacking this gene fail to generate GI slow waves [Hwang *et al.* 2009]. Further, expression of this
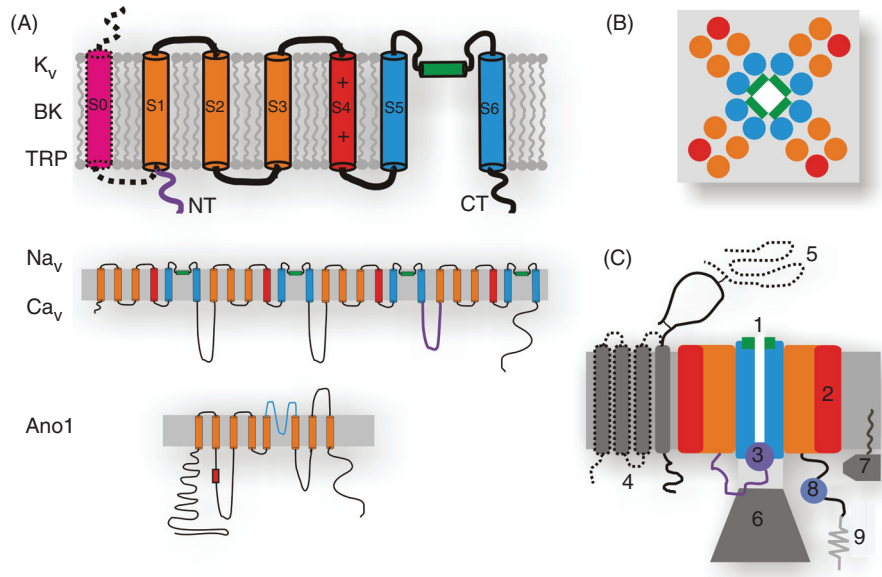
Figure 2. Fundamental aspects of the voltage-sensitive ion channel (VSIC) structures. (A) The building block of a VSIC is a six transmembrane (TM) subunit. The positively charged voltage sensor (S4, red) is supported in structure and function by S1-S3 (orange). The pore is lined by S5-S6 (blue) with P-loop being the selectivity filter (green). Big K (BK) channels have an additional S0 subunit (pink). The inactivation particle is formed by the N-terminus (purple). Potassium (K_V), BK and transient receptor potentials (TRPs) are tetramers, while sodium (Na_V) and calcium (Ca_V) channels are monomers with four linked homologous domains. Ano1 is structurally unique, having eight TMs with a pore region between TM 5 and 6, and a proposed voltage sensor on the intracellular loop between TM 2 and 3. (B) Top view of the VSIC of the K^+, Ca^+ and Na^+ families folds into a cruciform shape with the four S5-6 forming the pore and four voltage sensor domains (S1-S4) in the periphery making substantial contact with the bilayer lipids. (C) Side view of the VSIC modulatory elements that may serve as targets: (1) selectivity filter and C-type inactivation, (2) voltage sensors, (3) N-type inactivation, (4) Na_V β-subunit (solid gray), Ca_V γ-subunit (solid+dotted), (5) Ca_V α2-subunit, (6) K_V β-subunit, (7) KChIP, (8) calmodulin (CAM)-binding and EF-hand for Ca^{++} binding, (9) cytoskeleton binding via PDZ (on C-terminus) and ankyrin (DII-DIII) linker domains for Na_V1.5 for example.

protein in heterologous systems results in voltage- and calcium-sensitive Cl^- conductances [Schroeder et al. 2008] (Figure 3C). However, the situation is complex, since the topology map for these molecules lacks the traditional features of the voltage-sensitive channels [Sheridan et al. 2011], extensive alternative splicing alters sequence and function [Mazzone et al. 2011; Ferrera et al. 2009] and modulation by various stimuli makes these channels difficult to study [Tian et al. 2011].

Voltage-sensitive sodium selective channels
Various voltage-sensitive sodium selective channels. These channels are found in both the ICCs and SMCs of the circular smooth muscle layer of the human jejunum [Strege et al. 2003b; Holm et al. 2002; Ou et al. 2002], and dog [Strege et al. 2007], rat ileum [Smirnov et al. 1992] and gastric fundus [Muraki et al. 1991] as well as from the SMCs of rat and human colons [Xiong et al. 1993]. Na_V channels are highly voltage sensitive

inwardly rectifying with activation and inactivation kinetics on a millisecond scale (Figure 3D). Blocking of Na_V channels by lidocaine and QX-314 reduced the rate of slow wave rise and increased slow wave duration, resulting in a decrease of slow wave frequency [Strege et al. 2003b]. Both TTX-sensitive [Smirnov et al. 1992] and TTX-resistant [Holm et al. 2002] Na_V channels have been reported, suggesting that multiple Na_V isoforms may be present in the GI SMCs and ICCs although it appears that the dominant channel is the TTX-resistant Na_V1.5. The TTX-resistant current in human jejunum circular SMCs has been identified as Na_V1.5, which is typically referred to as the 'cardiac' sodium channel [Ou et al. 2002]. It was found that patients with mutations in the SCN5A gene, which codes for Na_V1.5, have more abdominal symptoms than controls. This finding was not accounted for by a prolonged QT interval [Locke et al. 2006]. SCN5A mutations are also prevalent in IBS [Saito et al. 2009b;
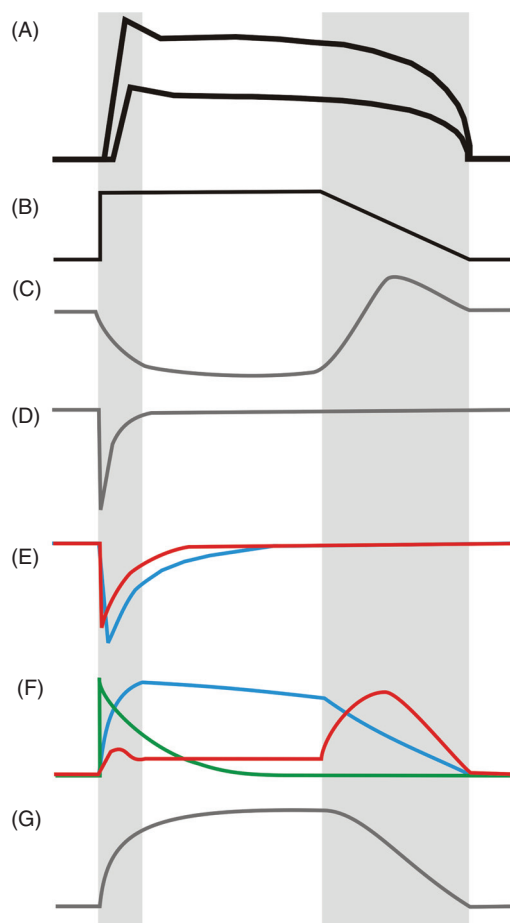
Figure 3. Cartoon of voltage-sensitive ion channel conductances involved in formation of ICC and SMC slow waves. (A) Generic ICC and SMC slow waves with gray bars highlighting upstroke and repolarization. (B) A simplified 'slow wave' voltage step protocol (from a holding potential of about -80 mV to 0 mV). Individual currents are shown in (C)-(G), with deflections downward and upward being inward (depolarizing), and outward (repolarizing) currents, respectively. (C) Ano1, (D) Na$_V$ currents, (E) Ca$_V$ currents showing Ca$_V$1 or L-type (blue), Ca$_V$3 or T-type (red), (F) potassium currents showing K$_V$3 and K$_V$4 or A-type (green), K$_V$1 or delayed rectifier and K$_{Ca1}$ or BK (blue), KV11.1 or ERG (red), (G) TRPM nonselective cation current.

Braak *et al.* 2008]. While the mechanism is not yet clear, a SCN5A mutation from an IBS patient resulted in smaller Na$_V$1.5 currents and reduced mechanosensitivity [Saito *et al.* 2009a].

Nonselective cation channels
Gene chip studies show that TRPC1,2,4 and TRPM4,7 are expressed on ICC in mice [Chen *et al.* 2007]. Transient receptor potential (TRP) family channels are nonselective cation channels and are traditionally not considered to be in the VSIC family [Clapham, 2003]. However, we include them in this discussion because they have similar topology to the classical voltage-gated ion channels, TRPC (classic) and TRPM (melastatin) show substantial voltage sensitivity, and recent data suggest that TRPs may play a role in generation of slow waves although the

exact molecular identity is still unclear (Figure 3G). TRPM7 in mouse-cultured ICC was reported as critical for slow waves [Kim *et al.* 2005] and in another study TRP4 were thought to be responsible for Ca^{++} influx, without which slow waves were abolished [Torihashi *et al.* 2002]. However, these data have yet to be widely reproduced.

Voltage-sensitive calcium selective channels
Voltage-sensitive calcium (Ca$_V$) channels L-type (Ca$_V$1) and T-type (Ca$_V$3) have been described in GI SMCs and ICCs. **T-type Ca^{++} channels** are also known as the low-voltage-gated, dihydro-pyridine (DHP)-resistant channels. Ca$_V$3.2 (α1h) voltage-gated calcium selective ion channels were identified as nifedipine resistant and mibefradil sensitive Ca^{++} currents that modulate the

voltage-dependent Ca^{++} influx into the pacer units, activating them and entraining pacemaker response [Kim *et al.* 2002] (Figure 3E). The T-type channel blocker mibefradil decreases the slow wave amplitude [Hotta *et al.* 2007] and frequency [Kito *et al.* 2005]. These findings were also confirmed in the *cacna1h* knock-out mice [Gibbons *et al.* 2009]. The **L-type Ca^{++} channels** are also known as the high-voltage-gated, DHP-sensitive channels. The $Ca_V1.2$ ($\alpha1c$) channels are densely expressed in the ICCs and SMCs [Chen *et al.* 2007]. These channels activate slower than the T-type channels on depolarization but have a higher total conductance in SMCs (Figure 3E). They provide the bulk of Ca^{++} required to initiate excitation–contraction [Corrias and Buist, 2007]. L-type currents are also known to be present in the ICCs [Kim *et al.* 2002]. Studies show that block of the L-type current has no significant effects on the slow waves [Dickens *et al.* 1999] but abolishes SMC mechanical activity [Farrugia, 1996]. Thus, constipation is a common side effect of the DHP antihypertensive medications, which block L-type currents [Farrugia, 1999]. On the other hand, the ubiquity of Ca^{++} channels in the GI tract is exploited by a novel antispasmodic otilonium bromide known to block both the L-type and multiple T-type subunits [Strege *et al.* 2010, 2004]. Since otilonium bromide is a quaternary ammonium that does not become systemically absorbed it may be used in the gut without systemic side effects [Evangelista, 2004].

Voltage-sensitive and calcium-activated potassium selective channels

Potassium channels make up half of the ion channel superfamily, and voltage-sensitive K_V channels are the largest group [Wulff *et al.* 2009]. This rich diversity of K^+ channels is reflected in the wide variety of potassium channels detected in ICCs and SMCs of the GI tract [Vogalis, 2000]. With the intracellular K^+ concentration more than 10-fold higher than extracellular, K^+ channel opening typically produces an outward K^+ flux that drives the membrane potential in the negative direction toward K^+ reversal potential. Consequently, as a broad oversimplification, K^+ channels maintain resting potential, participate in the plateau current and repolarization of the slow wave [Farrugia, 1999]. GI SMCs and ICCs express a broad range of K^+ channels with multiplicity of functional properties described by variable kinetics and some with sensitivities for secondary modulation by Ca^{++} and auxiliary proteins. This variability of K^+ channels and currents in the GI tract is responsible for the flexibility of the plateau and repolarizing waveforms. In this review we focus on the six TM voltage sensitive K channels, and will refrain from discussion of the many two TM K channels, such as K_{ir}, which also have significant roles (K channels in smooth muscle are reviewed by Vogalis [2000]).

Voltage-dependent K^+ currents limit the upstroke, set the plateau and initiate repolarization (Figure 3F)

The upstroke of the slow wave is due to nonselective cation channels, Ca^{++}, Cl^- and Na^+ channels as described above, and the upstroke of the AP is due to Ca^{++} influx through the L-type Ca^{++} channels and calcium-mediated calcium release. A-type K^+ currents (I_A) the earliest to activate after onset of depolarization and are responsible for limiting the slow wave upstroke. Inactivation of I_A is of the order of a second [Hille, 2001], so these channels are also likely involved in part in the maintenance of the slow wave plateau phase [Barajas-Lopez *et al.* 1989]. In the GI tract, the locations and precise isoforms are variable, but there is good evidence that the K_V4 [Amberg *et al.* 2003], K_V3 [Ohya *et al.* 1997] families of channels are responsible for A-type current in the SMCs and perhaps ICCs [Parsons and Huizinga, 2010]. K_V4 channels have especially diverse structures and make connections with an assortment of intracellular regulators, which significantly customize their behavior [Birnbaum *et al.* 2004]. Temporally following I_A are slow delayed rectifier K^+ (I_K) currents and *ether-à-go-go*-related genes (eag). The channels responsible for I_K current are TEA and 4-AP sensitive and commonly expressed in excitable cells. These are further divided into the rapid (I_{Kr}) and the slow (I_{Ks}) delayed rectifiers. Multiple K_V1 family are expressed by GI SMCs, including $K_v1.1$ [Huizinga *et al.* 2004; Hatton *et al.* 2001], $K_v1.2$ [Hart *et al.* 1993], $K_v1.5$ [Overturf *et al.* 1994]. The delayed rectifier K_v1 subunits also have variable expression in the SMCs, and have an ability to form heterotetramers [Hatton *et al.* 2001]. This leads to a large variation of the on–off current kinetics observed experimentally and likely custom tailoring of the slow wave morphology. The eag code for voltage-sensitive K^+ channels of three subfamilies (K_V11 and K_V12) that include eag, elk (eag-like), and erg (eag-related gene), with a common structural EAG domain in the N-terminus that regulates

the rates of deactivation by binding to the S4–S5 linker [Schwarz and Bauer, 2004]. These channels have been found in SMCs of the esophagus [Akbarali *et al.* 1999], stomach [Ohya *et al.* 2002] and colon [Shoeb *et al.* 2003]. However, in the small intestine of a mouse ERG channels were detected of the ICC-MY but not on the SMC [Zhu *et al.* 2003]. ERG channels are inwardly rectifying ion channels with complex kinetics [Smith *et al.* 1996]. They inactivate quickly upon depolarization, then recover from inactivation upon repolarization, so they are likely involved in the repolarization phase of the slow waves [Schwarz and Bauer, 2004; Zhu *et al.* 2003]. ERG block by E-4031 and cisapride resulted in slowing of the slow wave activity [Akbarali *et al.* 1999]. hERG channels are notorious for complicating VSIC drug discovery efforts due to their promiscuous binding pocket that leads to inhibition of cardiac hERG, prolonging repolarization and predisposing to arrhythmias [Wulff *et al.* 2009]. GI prokinetic drug cisapride ($5HT_4$ receptor agonist) was taken off the market due to its affinity for hERG [Rampe *et al.* 1997], but other more selective 5HT4 agonists are being designed specifically to minimize cross reactivity with hERG [Camilleri *et al.* 2009]. On the other hand, the difference between the cardiac and gut hERG is a truncation by a hundred residues in the gut [Shoeb *et al.* 2003], which may allow gut-specific targeting.

Calcium-activated K channels (K_{Ca}) decrease excitability
K_{Ca} channels are classified based on their conductance: small (SK), intermediate (IK) and big (BK). All of these channel types have been described in the GI smooth muscle [Farrugia, 1999]. BK channels are the only channels with intrinsic voltage-dependence, while IK and SK are not voltage sensitive outright, but appear so due to the voltage dependence of Ca^{++} influx. IK and SK channels are widely expressed in the SMCs and ICCs as well as in fibroblast-like cells (SK3) [Fujita *et al.* 2003], but due to the lack of voltage sensitivity we abstain from further discussion. The BK or maxi-K_{Ca} channels ($K_{Ca}1.1$) are homotetramers, but structurally unique in that they have an additional transmembrane segment S0 and a long C-terminus that forms a calcium binding bowl [Wu *et al.* 2010] (Figure 2A). While these channels are encoded by a single gene (*slo1*, *KCNMA1*), they undergo extensive processing leading to over 100 splice variants [Hille, 2001]. BK channels are ubiquitous in excitable cells, including the SMCs [Wang *et al.* 2010] and ICCs [Zhu and Huizinga, 2008]. Block of BK by ChTx has little impact on resting voltage but allows hyper-excitability [Hong *et al.* 1997]. Alternatively, BK activation reduced contractility of ileum smooth muscle [Dela Pena *et al.* 2009], so BK activators may be reasonable options for spasmodic motility disorders. It has also been shown that NO, which is produced in several places in the GI tissue activates the BK channels, increasing K^+ influx and decreasing excitability [Zhu and Huizinga, 2008]. BK channels likely function as negative feedback regulators, assisting in repolarization and controlling excitability by primary modulation by Ca^{++} and secondary modulation by auxiliary subunits [Hagen *et al.* 2003], neural [Zhu and Huizinga, 2008] and mechanical [Wang *et al.* 2010] inputs.

Voltage-sensitive ion channels are viable targets for functional and motility disorders
VSICs are the essential players in the electrical and mechanical activity of the GI tract, and thus they are viable targets for functional and motility disorders. In the human genome VSICs constitute the largest group of signaling molecules after protein kinases and G-protein coupled receptors [Sharman *et al.* 2011]. Recent advances in electrophysiology and pharmacology are now being combined with the structural information from X-ray crystallography to provide specific molecular binding sites and conformational states that are worthy of pharmaceutical targeting [Cuello *et al.* 2010]. However, there are some difficulties in these approaches. Below, we begin with the limitations of targeting VSICs in GI functional and motility disorders and then provide potential solutions.

Complete understanding of the components of the slow wave and AP is lacking
At the most fundamental level, the development of rational drug targets is impeded by the lack of slow wave details and action potential mechanisms. A major difficulty is that dissociation of tissues required for identification and functional characterization of ion channels results in changes in the cells' electrical activity. Techniques such as single-cell polymerase chain reaction (PCR) [Ou *et al.* 2002] and laser capture microdissection [Ou *et al.* 2003] are useful for isolation and study of ion channels from single cells. What is more, there are well-described

differences in the ion channel types and isoforms across gut regions and species [Strege *et al.* 2007], which means that the database of the involved ion channels is extensive.

Multifactorial nature of the GI motility and functional disorders likely requires a combination of targets

Once we understand in detail the molecular components of the electrical activity and the functioning of the individual ion channels, we can begin to rationally address the prime targets for pharmaceutical intervention. At the present time, we only know that interventions to modulate channel function generally produce an altered cellular activity. However, most commonly those changes are difficult to predict and the outcomes at the tissue level are not clear. Thus, clear cut prime targets for particular motility disorders do not exist. We are beginning to understand which of the VSICs are indispensable for electrical activity. Even when prime VSIC targets are elucidated, their modulation would need to be state dependent and not an on–off type approach. In addition, given the multifactorial nature of motility disorders, successful therapeutic approaches are likely to be multifaceted, targeting the CNS, ENS and the effectors (SMCs and ICCs).

VSIC types are conserved across electrically active organs

The GI tract is electrically active and functionally similar to the other excitable tissues, such as the cardiac, neuronal, skeletal and other smooth muscles (e.g. bladder and blood vessels). There are obvious major differences in the roles for these organs, but from the standpoint of electrical function the distinction is only with respect to the resting voltage, amplitude and kinetics of the cyclical electrical activity. Owing to the similarities in functional requirements and the fact that the evolutionary tolerance of dysfunctional electrical systems is low, there is significant redundancy in the molecular components of the electrical systems. As the ion gradients have little variation, the upstroke of electrical activity (depolarization) involves Na_V and Ca_V channels, while the K_V channels are typically responsible for repolarization. The families of Na_V and K_V channels have several members, and there are some that are found exclusively in certain tissues, but all of the channels expressed in the GI tract tissue have been described elsewhere. This overlapping channel distribution means that drugs with systemic circulation targeted to GI tissues will likely also affect the same VSICs in other tissues.

VSICs have significant structural homology

A variety of voltage-sensitive ion channels exists. Drugs that have targeted VSICs have suffered from cross reactivity with other VSICs [Wulff *et al.* 2009]. As noted in Figure 2, there is significant structural conservation, which is one of the reasons for some cross reactivity for the drugs developed to target-specific channels. On the other hand, recent studies have shown that both the pores and voltage-sensor functional domains can confer their specific properties when spliced onto other channels [Alabi *et al.* 2007]. Thus, as for the other molecular targets even small perturbations in sequence [DeSimone *et al.* 2009], the surrounding lipid composition [Lundbaek *et al.* 2004] and mechanical state of the bilayer [Schmidt and Mackinnon, 2008] have been shown to significantly affect drug function.

While the limitations of voltage-gated ion channels as molecular targets in GI functional and motility disorders are real, there are potential solutions for drug developers to consider. We provide details of the approaches for targeting specific VSIC structural and functional motifs below.

Ion channel pores as drug targets

The pores of VSICs have specific ion selectivity (K^+, Na^+, Ca^+, Cl^-) and may therefore serve as specific drug targets. The best known is the selectivity filter and pore (green and blue, respectively in Figure 2) of a K^+ channel (KcsA) which balance the needs for high selectivity and rapid conduction [Doyle *et al.* 1998]. In this example, the selectivity filter selects K^+ ions, strips them of surrounding water molecules and ushers them through the selectivity filter (green) and then into a water-filled pore cavity for rehydration (blue) [Berneche and Roux, 2001]. The selectivity and pores of the other ion channels share some homology [Lipkind and Fozzard, 2000] and mechanism, but mutations of only single residues in the selectivity filter sometimes allow significant shifts in selectivity [Heinemann *et al.* 1992]. Permeation through the channels can be affected by either blocking the inner water-filled pore or by binding near the external face of the pore near the selectivity filter. Drugs such as TEA for K^+ channels wedge into the water-filled pore to block conduction. This process is similar to the natural fast-inactivation (N-type) of

these channels by the ball-and-chain intracellular block (purple in Figure 2) [del Camino *et al.* 2000]. While the block by these molecules is a physical obstruction of the water-filled inner vestibule, its structure in the Na$_V$, Ca$_V$ and K$_V$ is different enough that blockers generally do not cross react across families [Hille, 2001]. On the other side of the filter, the best known drugs that target the extracellular face of the pore are toxins such as TTX for Na$_V$ [Hille, 2001] and α-KTx for K$_V$ [Avdonin *et al.* 2000]. An alternative idea to affect permeation and the selectivity filter is through C-type (slow) inactivation, which requires restructuring of the selectivity filter such that ion flow is interrupted (green in Figure 2) [Cuello *et al.* 2010]. It is likely that a simple pore block is not a selective enough approach, since entire families of currents are affected and the effects are potentially devastating. In contrast, targeting the kinetics of both N- and C-type inactivation is an attractive approach to modulate permeation through the pore.

Voltage sensors as drug targets
The hallmark of VSICs is that opening and closing transitions are closely linked to changes in transmembrane voltage, often responding to changes of a few millivolts. The voltage-sensing mechanism deserves intense scientific attention, since modulation of the voltage sensor function allows elaborate regulation of ion channel function. In most simple terms, each subunit posses a voltage sensor which is a transmembrane helix (S4) with a few (2–5) positively charged arginine or lysine residues (red in Figure 2) and the supporting transmembrane segments (S1–S3; orange in Figure 2) [Long *et al.* 2005b]. The transmembrane voltage does work on the charged S4 helix displacing it across the lipid bilayer, and this movement is coupled to the swinging of the intracellular activation gate opening the pore and allowing ion conduction [Long *et al.* 2005a]. Venomous organisms have utilized the power of voltage-sensor modification [Escoubas and Rash, 2004]. Researchers have in turn used small peptide toxins from the venoms to study and specifically target the voltage sensors [Bosmans and Swartz, 2010]. Voltage sensor toxins are gating modifiers, essentially modulating the function of the voltage sensors, making voltage sensor operation either more or less stable. This is an overly simplified view. Detailed studies of the voltage sensor operation support a voltage-sensing mechanism that involves multiple conformational

states [Bezanilla, 2008]. Each of these states can serve as a target for therapeutic intervention.

Ancillary subunits as drug targets
The VSIC α-subunits form fully functional ion channels. However, ancillary subunits and other modifiers (e.g. kinases and calmodulin) play a large role in determining ion channel function *in vivo* (Figure 2C) [Abriel, 2007]. These proteins constitute possible drug targets, as there is a variety of isoforms whose expression is frequently tissue specific. There is also flexibility in the approach for modulation by regulating either the levels of these proteins or their binding and activity at the α-subunits.

Biophysical considerations for pharmacologic targeting of VSICs
An alternative to the approach of targeting structural motifs is one of targeting specific functional aspects. Ion channels exist in many states in addition to the *closed*, *open*, and *inactivated*. For example, the voltage-sensing mechanism described above actually involves multiple conformational states [Bezanilla, 2008] and each of these states can serve as a target for therapeutic intervention. Moreover, conformational shifts during gating alter the affinity of some drugs for their binding sites, known as the 'modulated receptor' hypothesis [Hille, 1977]. An example of modulated receptor action is mibefradil binding to T-type Ca^{++} channels [Bezprozvanny and Tsien, 1995], which allows differential targeting specific to the voltage state of the system. In a guarded receptor model, the drug binding site cycles between completely accessible and inaccessible as the channels open and close, respectively. Then drug effect is use dependent and this effect may be utilized in the treatment of arrhythmias. An example is lidocaine binding by Na$_V$1.5 during cardiac tachyarrhythmias [Nau and Wang, 2004], or perhaps a different hyperactive channel in gastric tachyarrhythmia [Lammers *et al.* 2008].

Mechano-electrical considerations
We now know that VSICs have an impact on the mechanical state of the surrounding membrane [Beyder and Sachs, 2009] and in return many of the VSICs are mechanically sensitive [Schmidt and Mackinnon, 2008]. Mechanically sensitive VSICs include those in the SMCs and ICCs [Kraichely and Farrugia, 2007], such as Na$_V$1.5 [Beyder *et al.* 2010; Strege *et al.* 2003b], L-type Ca^{++} [Kraichely *et al.* 2009; Lyford *et al.* 2002], BK [Wang *et al.* 2010; Qi

et al. 2005] and K_V1 and K_V4 [Laitko et al. 2006]. The molecular mechanisms for mechanical sensitivity by VSICs are being elucidated and likely involve voltage sensors [Beyder et al. 2010] and channel gates [Beyder and Sachs, 2009; Laitko et al. 2006]. Alteration in mechanical sensitivity may be responsible for pathology in a subset of IBS patients [Saito et al. 2009a] and thus may serve as another viable target for VSICs.

The detailed structural information revealed from crystallography or electron microscopy [Wang and Sigworth, 2009] continues to emerge and is now being combined into the body of biophysical knowledge to understand the mechanisms of VSIC function [Nishida et al. 2007]. Other experimental approaches aim to fill the gaps between the static structural information and electrophysiology. Examples are nuclear magnetic resonance (NMR) [Shah et al. 2006], electron para magnetic resonance (EPR) [Cordero-Morales et al. 2006], fluorescence resonance energy transfer (FRET) and other fluorescent approaches [Posson et al. 2005]. When integrated together those data may provide detailed targets for VSICs.

Defining other molecular domains in VSICs

There is Mounting evidence that ion channels are regulated at the genetic and epigenetic level [Shao et al. 2009] and by posttranslational modification [Akbarali et al. 2010]. Ion channels, including VSICs, are specifically spliced and levels are tightly managed in different tissues and pathologic states [Mazzone et al. 2011; Shi et al. 2011; Wang et al. 2010; Walker et al. 2001; Bielefeldt, 1999; Ohya et al. 1997]. Ion channel splicing and posttranslational modification often produces channels with altered function, whether due to alteration of the voltage-dependence properties, current selectivity and flux, or binding to the regulatory domains. The implications are twofold. First, custom processing of VSICs that alters functional performance on the molecular level also alters function at the cellular and tissue level. For example, hERG channels may be spliced differently in the gut and the heart, with the functional changes demonstrated in the gut hERG channels [Shoeb et al. 2003]. There is new evidence that alternative splicing of Ano1 in diabetic gastroparesis leads to functional consequences [Mazzone et al. 2011]. Second, such variations provide site- and disease-specific targets. An example of a successful splice targeting is the tissue-specific DHP block of the vascular L-type Ca^{++} channel due to alternative splicing of the DIS6 segments [Welling et al. 1997]. As our understanding of the alternative splicing mechanisms continues to advance other potential targets will emerge. These will allow control at the genetic and epigenetic levels as well as splice variant specific modulation by phosphorylation [Ahern et al. 2005] and interaction with G-proteins [Yarbrough et al. 2002].

Future directions

The continuous development of experimental tools for the study of electrophysiology in GI tissues has produced a deep understanding of electrical activity down to the molecular level. However, this progress has yet to bring the clinical tools that can diagnose and monitor GI motility disorders. In order to continue toward that goal we need to further our efforts to understand the mechanisms from the single molecule to the tissue levels, and then to build rational models and noninvasive ways to test those models. VSICs should figure prominently in this plan, since their function is vital to GI motility. In fact, their properties are a focus of intense study. Computational models are beginning to emerge that once populated with some experimental data on VSICs will be able to predict complex activity at the cellular and tissue levels [Faville et al. 2008; Corrias and Buist, 2007] (for a recent review see Lees-Green et al. [2011]). Furthermore, the development is continuing toward minimally invasive measurements of electrical activity in vivo [Egbuji et al. 2010]. These efforts may lead a state where for GI motility disorders we will be able to measure electrical activity in vivo and compare with the predicted normal, pointing out specific molecular pathologies and thus therapeutic targets.

Summary

Pathologies involving ICCs and SMCs lead to GI functional and motility disorders. Slow waves and action potentials are the signaling events that underpin normally functioning ICCs and SMCs. Voltage-sensitive ion channels are indispensable for slow waves and action potentials. Many of the voltage-sensitive ion channel species involved in the electrical activity of these two cell types have been discovered and their functions elucidated in remarkable but not yet sufficient detail. These molecules serve as potential exciting targets for pharmaceutical interventions.

Conflict of interest statement
The authors declare no conflicts of interest in preparing this article.

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