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Keeping Pace with Interstitial Cells of Cajal: Modelling Gastrointestinal Electrophysiology

Rachel Lees-Green

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in Bioengineering
Auckland Bioengineering Institute
The University of Auckland
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Abstract

Gastrointestinal (GI) motility is coordinated by several cooperating mechanisms, including myogenic, neural and hormonal control systems. This thesis focuses on one of these mechanisms: an intrinsic bioelectrical activity called slow waves, which originates in pacemaker cells called interstitial cells of Cajal (ICC) located within the smooth muscle layers of the GI tract. The mechanism by which ICC generate slow waves is a matter of ongoing research, although rhythmic calcium (Ca$^{2+}$) oscillations are known to underlie slow wave activity, and both voltage- and Ca$^{2+}$-dependent ion channels are involved in the pacemaker mechanism. In this thesis, mathematical models of pacemaker activity were developed to investigate the mechanisms by which slow waves are generated and regulated in ICC.

The literature was reviewed to determine the ion channels and Ca$^{2+}$ dynamics likely to contribute to ICC pacemaker activity, particularly the identity of the pacemaker channel that initiates the slow wave and the channels that contribute to the characteristic plateau phase of the slow wave. A pacemaker hypothesis was proposed in which the pacemaker channel is a Ca$^{2+}$-activated chloride (Cl$^{-}$) channel called anoctamin 1 (Ano1), which is activated by a localised increase in intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$). Cyclical release of Ca$^{2+}$ from intracellular stores is believed to initiate pacemaker activity, so Ano1 was proposed to be activated by Ca$^{2+}$ influx through store-operated Ca$^{2+}$ (SOC) channels.

A novel mathematical model of Ano1 current was constructed. The Ano1 model reproduced experimentally observed behaviour, including the steady-state voltage- and Ca$^{2+}$-dependent activation profile, slow activation at low [Ca$^{2+}$]$_i$, rapid activation at high [Ca$^{2+}$]$_i$, and slow deactivation when [Ca$^{2+}$]$_i$ is reduced.
The Ano1 model was then incorporated into a new compartmental model of small intestinal ICC pacemaker activity based on the proposed pacemaker hypothesis. A series of simulations were carried out using the ICC model to investigate current controversies about the reversal potential of the Ano1 Cl\(^-\) current in ICC, and to predict the characteristics of the other ion channels that are necessary to generate slow waves. The model showed that Ano1 is a likely pacemaker channel when coupled to a SOC channel, but predicted that ICC in Ano1 knockout mice may still generate small cyclical depolarisations despite the absence of the pacemaker channel. The results suggested that voltage- or Ca\(^{2+}\)-activated non-selective channels or sodium (Na\(^+\)) channels may contribute to the slow wave plateau phase, whereas voltage-dependent Ca\(^{2+}\) current is likely to be negligible during the plateau. The Cl\(^-\) equilibrium potential was shown to be an important modulator of slow wave morphology, highlighting the need for a better understanding of Cl\(^-\) dynamics in ICC in order to clarify how Ano1 and other Cl\(^-\) currents contribute to the slow wave plateau and repolarisation.

The spontaneous pacemaker activity of ICC is also regulated by mechanical inputs. An original model of a mechanosensitive Na\(^+\) channel found in human small intestine ICC was developed and incorporated into a previously published small intestine ICC model. Simulation results showed that mechanosensitive changes in the Na\(^+\) current caused up to 5\% depolarisation of resting membrane potential, 11\% increase in slow wave upstroke rate, 5\% increase in slow wave duration, and 1\% increase in frequency. These results were comparable to the experimentally observed effects of stretching smooth muscle tissue, indicating that Na\(^+\) channel mechanosensitivity can explain the effects of stretch on slow waves.

In summary, this thesis presents: a new model of Ano1 current; the first ICC model to implement Ano1 as a pacemaker channel and to include store-operated Ca\(^{2+}\) entry as a component of the pacemaker cycle; and the first model of slow wave regulation by mechanical stimuli.
Acknowledgements

The completion of this thesis was possible thanks to the help of a number of people.

First and foremost, I would like to acknowledge Andrew Pullan for introducing me to the fascinating world of interstitial cells of Cajal, for providing me with the opportunity to do a PhD, and for all the advice and support he gave me as a supervisor and mentor. Andrew sadly passed away during the course of my PhD, but his passion for research and his unique outlook on life continue to inspire me.

I greatly appreciate the support I have received from my supervisors, James Sneyd and Leo Cheng. Thank you to James for being so supportive and encouraging, and for providing excellent advice and guidance about modelling cells. Thank you to Leo for being exceptionally helpful, particularly for assistance with the administrative aspects of my PhD and thorough help with proof-reading.

I am grateful to Gianrico Farrugia, Art Beyder, and Simon Gibbons at the Mayo Clinic for providing insight into the messy side of biomedical research.

To my fellow students and post-docs in both the GI and Calcium groups, thank you for sharing the ups and downs of the PhD journey with me. Special thanks to Peng Du, Greg O’Grady and Shawn Means for lively discussions and advice.

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<td>9-anthracene carboxylic acid</td>
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<td>Ano1</td>
<td>anoctamin 1, calcium-activated chloride channel</td>
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<td>large conductance or Maxi-K calcium-activated potassium channel</td>
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<td>$\text{Ca}^{2+}$</td>
<td>calcium ion</td>
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<td>cpm</td>
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<td>EC$_{50}$</td>
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<td>$E_{\text{Ca}}$</td>
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<td>GFP</td>
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<td>gastrointestinal</td>
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<td>HEK 293</td>
<td>human embryonic kidney 293 cells</td>
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<td>ICC model variation with $E_{Cl}$ set to $-20,\text{mV}$</td>
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<td>IC$_{50}$</td>
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<td>I$_{CaV}$</td>
<td>voltage-dependent calcium current</td>
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<tr>
<td>ICC</td>
<td>interstitial cell(s) of Cajal</td>
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<td>ICC-CM</td>
<td>ICC located within the circular smooth muscle</td>
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<td>ICC located in the region of the deep muscular plexus</td>
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<td>ICC located within the smooth muscle layers</td>
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<td>voltage-dependent non-selective current</td>
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<td>IP$_3$</td>
<td>inositol 1,4,5-trisphosphate</td>
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<td>IP$_3$ receptor</td>
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<td>K$^+$</td>
<td>potassium ion</td>
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<td>KCNK3</td>
<td>gene encoding the K$_{2P3.1}$ two-pore domain background potassium channel</td>
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<td>$K_d$</td>
<td>dissociation constant</td>
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<td>sodium/calcium exchanger</td>
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<td>nickel ion</td>
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<td>NSC</td>
<td>non-selective cation (channel or current)</td>
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<td>ODE</td>
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<td>PMCA</td>
<td>plasma membrane calcium ATPase</td>
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<td>PMU</td>
<td>pacemaker unit, also abbreviated as PU</td>
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<td>$R$</td>
<td>universal gas constant, $8.314,\text{J},\text{mol}^{-1}\text{K}^{-1}$</td>
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<td>STIC</td>
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<td>$V_m$</td>
<td>membrane potential</td>
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<td>W/W$^\text{v}$</td>
<td>mice with a mutation in the white spotting (W) locus that encodes the Kit gene</td>
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Chapter 1

Introduction

The foundations of electrophysiology were laid in the 18th century when Luigi Galvani made severed frogs’ legs contract by stimulating their nerve fibres with electricity or with the cut end of another nerve (Galvani, 1791). Many years of elaborate experiments led Galvani to develop the theory that an intrinsic ‘animal electricity’ activated muscle contractions. The notion that electricity could reanimate dead body parts later captured the public imagination and inspired Mary Shelley’s *Frankenstein* after Galvani’s nephew, Giovanni Aldini, demonstrated the animating effects of electricity on the face and limbs of recently executed criminals (Bresadola, 1998; Piccolino, 1998; Verkhratsky et al., 2006).

Like all good science, Galvani’s experiments raised more questions than answers, and a thorough understanding of animal electricity awaited the development of modern instruments and experimental techniques. We now know that intrinsic bioelectricity in the nerves and muscles of humans and other animals arises from the unequal accumulation of charged ions on either side of an insulating membrane enclosing the cell. Electrical signals are generated when ion channels in the membrane open or close, allowing ions to flow through the water-filled pores of the channels.

The ionic basis of cell excitation was demonstrated by Alan Hodgkin and Andrew Huxley using intracellular electrodes on the giant axons of squid. Their research into the voltage sensitivity and kinetics of ion currents in nerve cells culminated in the first mathematical model of membrane action potentials (Hodgkin and Huxley, 1952), which set the benchmark for subsequent quantification and modelling of cell excitability (Schwiening,
1. INTRODUCTION

Hodgkin and Huxley won a share of the Nobel Prize in Physiology or Medicine in 1963 “for their discoveries concerning the ionic mechanisms involved in excitation and inhibition in the peripheral and central portions of the nerve cell membrane” (Nobel Media AB, 2013).

Electrical signals in excitable cells must elicit a non-electrical response to be useful, such as the contraction of muscle fibres or the release of neurotransmitters at a synapse. This typically occurs when voltage sensitive calcium (Ca\(^{2+}\)) channels open or close, changing the Ca\(^{2+}\) concentration within the cell (Hille, 2001). As an example, the contraction of the skeletal muscles in Galvani’s frog legs occurred because an electrical signal that travelled from the nerve fibre to the muscle caused an increase in intracellular Ca\(^{2+}\) concentration, which activated the contractile machinery in the muscle fibres.

A similar process leads to contraction in all types of muscle, including smooth muscle cells in the gastrointestinal (GI) tract. Contractions of the GI tract wall contribute to digestion by mixing, grinding and transporting food, but unlike in skeletal muscle, these smooth muscle contractions are not exclusively controlled by electrical signals from the nervous system. Instead they are partly controlled by spontaneous electrical events called slow waves, which originate from pacemaker cells called interstitial cells of Cajal (ICC) located within the muscular layers of the GI tract (Farrugia, 2008). The research presented in this thesis used the mathematical modelling techniques pioneered by Hodgkin and Huxley (1952) to investigate the mechanism by which ICC generate electrical pacemaker activity in the GI tract.

1.1 Motivation and Aims

Understanding the origin of slow waves is a key aspect of GI research, but exactly how ICC generate slow waves remains unresolved (Mawe, 2009). The identification of ICC pacemaker function occurred relatively recently (Huizinga et al., 1995; Ward et al., 1994), and difficulties associated with recording directly from ICC have hindered efforts to elucidate their pacemaker mechanism (Lees-Green et al., 2011b). Research has shown that cyclical changes in intracellular Ca\(^{2+}\) underlie slow wave activity in ICC (Rich et al.,
2002; Torihashi et al., 2002), but the roles of ion channels identified in ICC and the exact mechanism of interaction between the intracellular Ca\(^{2+}\) dynamics and the ion currents contributing to the slow wave potential remain a matter of ongoing discussion (Beyder and Farrugia, 2012; van Helden et al., 2010). The identity of the pacemaker channel that initiates slow waves is particularly contentious, as several candidates have been proposed, but none are fully compatible with what is known of the pacemaker mechanism in ICC (Farrugia and Kraichely, 2005; Huizinga and Chen, 2014; Mawe, 2009).

A likely candidate pacemaker channel is a Ca\(^{2+}\)-activated chloride (Cl\(^{-}\)) channel called anoctamin 1 (known as Ano1 in mice or ANO1 in humans). The function of Ano1 as a Ca\(^{2+}\)-activated Cl\(^{-}\) channel was discovered in 2008 (Caputo et al., 2008; Schroeder et al., 2008; Yang et al., 2008), and the following year, Ano1 was found to be expressed in all classes of ICC within the human and mouse GI tracts (Gomez-Pinilla et al., 2009). Ano1 knockout mice do not exhibit slow waves, indicating that Ano1 is essential for the generation of electrical pacemaker activity (Hwang et al., 2009).

It is important to test whether the Ano1 channel can function as a pacemaker channel, and mathematical modelling provides an ideal means to do this. Mathematical models can integrate experimental results to test hypotheses and explore aspects of cell function that are not easy to investigate experimentally. However, previous models of ICC pacemaker activity were published before the discovery of Ano1 in ICC and do not adequately reflect the present understanding of ICC pacemaker mechanisms (Corrias and Buist, 2008; Faville et al., 2009; Means and Sneyd, 2010; Youm et al., 2006).

In addition, the previous models do not handle inputs such as mechanical stimuli and neural signals, which are known to modulate the intrinsic pacemaker activity of ICC (Huizinga and Lammers, 2009; Iino and Horiguchi, 2006; Kraichely and Farrugia, 2007). Developing models of these regulatory mechanisms is an essential step towards an improved understanding of GI electrophysiology.

Gaining a better understanding of the complex processes involved in generating normal pacemaker activity will enhance our ability to identify how pacemaker activity is altered in GI disorders. ICC defects are implicated in both acquired and congenital GI motility disorders (Huizinga et al., 2009). For example, loss or injury of ICC is commonly associated
with gastroparesis or delayed gastric emptying (Grover et al., 2011), and mutations in a sodium (Na$^+$) channel that contributes to ICC pacemaker activity have been linked to GI symptom complexes, including irritable bowel syndrome (Locke et al., 2006; Saito et al., 2009). Therefore, changes in the pacemaker function of ICC are likely to contribute to the symptoms of these disorders.

The social and economic burden of GI disorders makes it imperative to find suitable treatments. Irritable bowel syndrome has an estimated prevalence of 4–22% in Western countries (Drossman et al., 2002), and has a high socioeconomic cost resulting from healthcare costs, work absenteeism, and reduced quality of life for patients (Maxion-Bergemann et al., 2006). Gastroparesis is less common among the general population, but 48% of diabetic patients have delayed gastric emptying (Jones et al., 2001), and 12–18% of diabetics have upper GI symptoms such as nausea, vomiting, or early satiety (Bytzer et al., 2001; Enck et al., 1994). The increasing prevalence of diabetes in the United States contributed to an 158% increase in gastroparesis-related hospitalisations from 1995 to 2004 (Wang et al., 2008b). Advancing our understanding of the changes underlying disorders like irritable bowel syndrome and gastroparesis will pave the way for better treatment options for patients (Hulisz, 2004).

Taking into consideration the aforementioned issues, the aims of this thesis were to:

1. Develop a hypothesis of how ICC generate slow waves based on a comprehensive review of the components and processes that underlie ICC pacemaker activity;

2. Identify existing mathematical models of ICC function and evaluate their ability to reproduce the proposed hypothesis;

3. Construct a model of the proposed pacemaker channel, namely the Ano1 Ca$^{2+}$-activated Cl$^-$ channel;

4. Develop an updated ICC model incorporating the Ano1 channel model to represent the proposed pacemaker hypothesis;

5. Investigate how mechanical stimuli in the GI tract modulate slow waves in ICC.
1.1. Motivation and Aims

The structure of this thesis is as follows: Chapter 2 presents an overview of the history of ICC research, followed by a summary of the methods used to study ICC and their strengths and limitations. The chapter then reviews the Ca\(^{2+}\) handling processes, ion channels and electrical events observed in ICC, and hypothesises how these components and processes may combine to generate ICC pacemaker activity.

In Chapter 3, the methods used to model electrical activity in the GI tract are briefly reviewed. Previously published ICC models are presented, and their strengths and shortcomings are considered. The pacemaker mechanism underlying the previous models is then compared with the pacemaker hypothesis proposed in Chapter 2, and the need for a new model of ICC activity is discussed in Section 3.4.

Chapter 4 focuses on ion channel mechanosensitivity in ICC. A model of a mechanosensitive Na\(^+\) channel is developed and incorporated into a previous model of small intestine ICC (Faville et al., 2009). The model is used to investigate the impact of mechanical stimuli on ICC slow wave activity.

A model of the Ano1 channel is presented in Chapter 5 and validated against experimental data.

In Chapter 6, the Ano1 model is then incorporated into a new model of ICC pacemaker activity based on the pacemaker hypothesis proposed in Chapter 2. The new ICC model is used to explore how changes in ion currents affect the proposed pacemaker mechanism. In particular, variations in Cl\(^-\) and Ca\(^{2+}\) currents are shown to alter the viability of the pacemaker hypothesis.

Finally, Chapter 7 summarises the key findings of this thesis and considers important directions for future research.
Chapter 2

Gastrointestinal Electrophysiology


Food is the fuel of life for humans and other animals, providing the energy and molecular building blocks that we need in order to function and thrive. In order to make good use of the food we eat, our digestive system mechanically and chemically breaks food down, absorbs nutrients and water, excretes waste, and protects the body from digestive enzymes and pathogens. These processes occur in the GI tract, which is essentially a continuous tube that runs from the mouth to the anus, divided into four organs: oesophagus, stomach, small intestine, and large intestine or colon (see Figure 2.1). The small intestine is divided into three sections: duodenum, a short section connected to the stomach; jejunum; and ileum, connected to the colon.

GI motility, the mechanical mixing and transport of contents along the gut, is essential for the healthy functioning of the digestive system. Despite more than a century of research into patterns and mechanisms of GI motility (Bayliss and Starling, 1899; Cannon, 1902), many aspects of the processes underlying motility are still not well understood (Farrugia, 2008; Huizinga and Chen, 2014; Sanders, 2008).

2.1 Electrical Control of Gastrointestinal Motility

The coordinated smooth muscle contractions that cause GI motility are controlled in part by spontaneous electrical events called slow waves in combination with regulatory neural
Figure 2.1: The human gastrointestinal system showing the major organs involved in digestion along the gastrointestinal tract. Adapted from Villarreal (2008).
and hormonal inputs (Grundy and Brookes, 2012; Huizinga and Lammers, 2009). Slow waves originate in pacemaker cells called ICC located within the muscle layers of the GI tract. Networks of ICC propagate slow waves to smooth muscle cells (SMC) where they can stimulate contraction. Intracellular recordings of depolarisations in pacemaker ICC have a larger amplitude than those in SMC, as shown in Figure 2.2, and electrical events recorded directly from ICC are commonly called pacemaker potentials to distinguish them from the lower amplitude slow waves recorded from SMC.

![Figure 2.2: Typical intracellular recordings showing pacemaker potentials from an ICC and slow waves from a smooth muscle cell from mouse small intestine. Reproduced from Lees-Green et al. (2011b).](image)

The resting membrane potential (RMP) in both ICC and SMC is typically $-80$ to $-55$ mV. Pacemaker potentials depolarise the membrane potential of ICC to a peak potential of $-25$ to $0$ mV, while slow waves in SMC have a peak potential of $-40$ to $-25$ mV (e.g. Dickens et al., 1999; Kito and Suzuki, 2003; Sanders et al., 2006). Slow wave
depolarisations shift the SMC membrane potential from a region of low open probability for voltage-dependent Ca$^{2+}$ channels to a potential with increased channel open probability, thereby facilitating smooth muscle contractions (Sanders et al., 2006). Although slow waves alone can cause sufficient Ca$^{2+}$ influx to initiate contractions (Ozaki et al., 1991), functionally significant contractions are a consequence of co-regulatory conditions being met, as occurs following a meal (Huizinga and Lammers, 2009).

2.2 Interstitial Cells of Cajal

2.2.1 Searching for a Purpose

The journey to the discovery that ICC act as pacemakers in the GI tract began in the late 19th century, when Santiago Ramón y Cajal, commonly held to be the father of modern neuroscience, described branching networks of the nerve-like interstitial cells that we now know as ICC (Cajal, 1911). Cajal created detailed drawings of the cells he saw under his microscope, such as those in Figure 2.3. However, the lack of specific markers for ICC hindered efforts to determine their function for many decades. For much of the 20th century, ICC were widely considered to be a terminal component of the peripheral nervous system, before an altered understanding of the structure of nerves resulted in ICC being downgraded in the literature to mere connective tissue (as reviewed by Thuneberg, 1999). The development of tissue preparation and electron microscopy techniques in the latter half of the century led to the observation that ICC were closely associated with both neurons and SMC, suggesting that innervated ICC transmit neural signals to SMC (Imaizumi and Hama, 1969; Stach, 1972; Yamamoto, 1977). Interest in ICC began to increase, and Thuneberg (1982) sparked a revival in ICC research by proposing that ICC are intestinal pacemaker cells. In support of this hypothesis, selective injury of ICC stained with methylene blue was shown to abolish slow wave activity (Liu et al., 1994; Thuneberg et al., 1983).

The real breakthrough in ICC research was the discovery that blocking the function of the receptor tyrosine kinase Kit protein during development causes GI abnormalities by
impeding the development of the pacemaker system (Maeda et al., 1992). Studies showed that W/WV mice, which are heterozygous for a mutation in the white spotting (W) locus that encodes the Kit gene (Chabot et al., 1988; Geissler et al., 1988), had a significantly reduced ICC population and an absence of electrical slow wave activity, but no apparent changes in neural pathways or SMC, indicating the importance of ICC for pacemaking (Ward et al., 1994; Huizinga et al., 1995). Subsequent electrical recordings from cells identified as ICC in the guinea-pig gastric antrum confirmed that ICC act as pacemakers in the GI tract (Dickens et al., 1999). The availability of Kit as a specific marker for ICC (Torihashi et al., 1995) motivated significant advancements in ICC research over the last two decades.

### 2.2.2 Beyond the Gastrointestinal Tract

Following the discovery that ICC act as pacemakers in the GI tract, ICC-like cells have been discovered in many other organs, including in the urinary tract (Davidson and McCloskey, 2005; Metzger et al., 2005; Sergeant et al., 2000; Shafik et al., 2004), the female reproductive
system (Allix et al., 2008; Gherghiceanu and Popescu, 2005; Popescu et al., 2007), the male reproductive system (Exintaris et al., 2002; Hashitani and Suzuki, 2004; Van der Aa et al., 2003) blood vessels (Bobryshev, 2005; Harhun et al., 2004), and lymphatic vessels (Briggs Boedtkjer et al., 2013; McCloskey et al., 2002). In addition, ICC-like cells have been found in the gall-bladder (Pasternak et al., 2013), pancreas (Popescu et al., 2005), and extrahepatic bile duct (Ahmadi et al., 2010) within the digestive system, and have even been found in the heart (Kapa et al., 2010; Kostin and Popescu, 2009).

These ICC-like cells have diverse functions, including generating, modulating, and propagating electrical activity. Their similarities to ICC in the GI tract, such as close association with SMC and nerves (Davidson and McCloskey, 2005; Hutchings et al., 2009) and evidence of spontaneous rhythmicity in some ICC-like cells (Harhun et al., 2004; Sergeant et al., 2000), have led to speculation that ICC-like cells act as pacemakers (Exintaris et al., 2002; Lavoie et al., 2007; McCloskey et al., 2002). However, definite evidence of a pacemaker function analogous to that in ICC has not yet emerged. In the urethra, ICC-like cells may act as ‘loose’ pacemakers by providing random signals to maintain muscle tone (Hashitani and Suzuki, 2007), while in other parts of the urinary tract they probably regulate pacemaker activity that originates in typical and atypical SMC (McCloskey, 2011). Similarly, ICC-like cells in the uterus appear to modulate contractility (Allix et al., 2008). In the uterus and gall-bladder there is evidence that ICC-like cells coordinate and propagate electrical activity (Hutchings et al., 2009; Lavoie et al., 2007). In human pulmonary veins, ICC-like cells may be associated with atrial fibrillation (Morel et al., 2008).

2.2.3 Different Populations of ICC

Experimental evidence suggests that ICC have multiple distinct roles in the GI tract in addition to their role as pacemaker cells, and the different functions are associated with different classes of ICC. Several types of ICC have been classified by location, as shown in Figure 2.4. ICC located in the region of the myenteric nerve plexus between the
longitudinal and circular smooth muscle layers, are called ICC-MY,\(^1\) although ICC-MY are not found in the gastric fundus of small animals (Hanani et al., 2005). Intramural ICC (ICC-IM) within the muscle layers are further sub-classified as ICC-CM within the circular muscle layers, ICC-LM in the longitudinal muscle layer, ICC-DMP in the deep muscular plexus between circular muscle layers, and ICC-SEP identified in the septa between circular muscle bundles (Hanani et al., 2005; Horiguchi et al., 2001). ICC-LM and ICC-CM are sparsely distributed in the small intestine, particularly in small animals. Submucosal ICC are located at the submucosal border of the circular muscle layer in the stomach (ICC-SM) and in the region of the submucosal plexus in the colon (ICC-SMP) (Horiguchi et al., 2001; Rumessen et al., 1993). Finally, ICC-SS are found in the subserosal layer adjacent to the longitudinal muscle layer (Hanani et al., 2005).

ICC-MY are the primary pacemaker cells in the stomach and small intestine (Dickens et al., 1999; Hirst and Ward, 2003), while ICC-SMP are considered the primary pacemakers in the colon (Yoneda et al., 2002), although ICC-IM may also act as pacemakers in the guinea-pig stomach (Hashitani et al., 2005). Rapid propagation of slow waves through electrically coupled pacemaker ICC networks is essential for coordinating smooth muscle contractions on a macroscopic scale, so multipolar pacemaker ICC are connected in branching networks. ICC-CM actively regenerate slow waves in circular muscle (Dickens et al., 2001; Horiguchi et al., 2001), and in large animals ICC-SEP conduct slow waves from ICC-MY deep into circular muscle bundles (Lee et al., 2007a). ICC-IM, including ICC-DMP, are responsible for mediating neural signals from the enteric nervous system, particularly cholinergic and nitrergic neurotransmission (Iino and Horiguchi, 2006; Suzuki et al., 2003). Excitatory vagal stimulation can also lead ICC-IM to become primary pacemakers (Hirst et al., 2002b).

\(^1\)The myenteric plexus is also known as Auerbach’s plexus or rarely the myenteric ganglionated plexus, so ICC-MY have also been called ICC-MP, ICC-AP, or even ICC-MGP (Faussone-Pellegrini and Thuneberg, 1999; Thuneberg, 1999).
2. GASTROINTESTINAL ELECTROPHYSIOLOGY

Figure 2.4: Schematic of the location of ICC within the different layers of the GI tract. ICC-SM and ICC-SMP are the innermost ICC, located at the border of the submucosa and circular muscle. ICC-DMP occur between circular muscle layers in the small intestine. ICC-CM and ICC-LM are located within circular and longitudinal muscle, respectively. ICC-MY (ICC-MP) are located between the circular and longitudinal muscle layers. ICC-SS are the outermost ICC, located within the subserosa. Reprinted from Hanani et al. (2005), with permission from Elsevier.
2.2.4 Structural Features of ICC

ICC have two to five primary processes that, in mouse small intestine, are 100µm or more in length, and which branch to give secondary and tertiary processes, as shown in the drawings of ICC in Figures 2.3B and 2.4 (Faussone-Pellegrini and Thuneberg, 1999; Rumessen and Vanderwinden, 2003). Classes of ICC that form reticular networks, notably ICC-MY and ICC-DMP, are stellate cells, whereas ICC-CM and ICC-LM are bipolar spindle-shaped cells aligned in the direction of the circular and longitudinal smooth muscle fibres, respectively. The propagation of slow waves through ICC networks and into the surrounding smooth muscle tissue depends on ICC–ICC and ICC–SMC coupling. ICC within networks are connected to each other via gap junctions. ICC-IM (including ICC-DMP) also form gap junctions with SMC, which may play a role in the neurotransmission function of these ICC (Hanani et al., 2005; Thuneberg, 1999). However, ICC-MY do not form gap junctions with SMC, and how networks of ICC-MY communicate with other classes of ICC is not well understood (Thuneberg, 1999).

An alternative coupling mechanism for ICC and SMC involves peg-and-socket junctions, in which pockets of the SMC plasma membrane act as ‘pegs’ inserted into ‘sockets’ in the ICC membrane (Huizinga et al., 2010; Thuneberg, 1999). Electrical coupling may occur via electric field potentials generated between closely apposed membrane sections (Daniel, 2004), possibly in in peg-and-socket junctions (Vigmond et al., 2000).

Transmission electron microscopy (TEM) images have been used to identify both gap junctions and peg-and-socket junctions between ICC and neighbouring cells (Hanani et al., 2005; Thuneberg, 1999). TEM has also been used to identify a number of defining ultrastructural features of ICC, which are shown in Figure 2.5. Of particular note is the observation that ICC contain an abundance of mitochondria throughout the cytoplasm, and an extensive network of smooth endoplasmic reticulum (ER),\(^2\) including many sections near the cell surface, often in close proximity to invaginations of the plasma membrane called caveolae. Other ultrastructural features observed in both human and mouse ICC include actin microfilaments (thin filaments), intermediate filaments, and microtubules—all

\(^2\)Smooth ER is, amongst other functions, responsible for storing and releasing Ca\(^{2+}\) ions.
Figure 2.5: Ultrastructural features of ICC-MY (IC), including mitochondria (mit); smooth and rough endoplasmic reticulum (SER and RER, respectively); caveolae; basal lamina; and thin and intermediate filaments. The relationship between ICC and other cells is also shown, including the close association between ICC-MY and nerves; peg-and-socket junctions (psj) with longitudinal muscle (LM) and circular muscle (CM) cells; and a gap junction (gj) between two ICC. Reproduced from Faussone-Pellegrini and Thuneberg (1999) with permission from John Wiley and Sons.
components of the cellular cytoskeleton; and a form of extracellular matrix called basal lamina (Faussone-Pellegrini and Thuneberg, 1999; Rumessen and Vanderwinden, 2003).

### 2.2.5 Regulating Motility

ICC can generate slow waves independently of any neural inputs (Ward et al., 1999), but the control of GI motility by intrinsic slow wave activity is complemented by neural and hormonal regulatory systems (Huizinga and Lammers, 2009; Sanders, 2008). Non-pacemaker ICC act as intermediaries between neurons and SMC. Signals from both the intrinsic enteric nervous system and the extrinsic central nervous system modulate the generation of slow waves by ICC and control sensitivity of the smooth muscle contractile apparatus to depolarisation. The enteric nervous system can act autonomously, hence its status as the ‘second brain’. However, the central nervous system monitors the state of the GI system, providing conscious sensation and subconscious reflexes, and modulates enteric neural activity (Grundy and Brookes, 2012).

Slow waves and motility can also be modulated by mechanical stimulation. Mechanosensitivity in the gut is in part mediated by the enteric and central nervous systems (Grundy and Brookes, 2012). However, ICC have intrinsic mechanosensitive components too, and can act as stretch sensors in addition to the roles of slow wave generation, propagation, and neural mediation described above (Won et al., 2005). For example, both ICC and SMC in the GI tract contain stretch sensitive ion channels (Lyford et al., 2002; Strege et al., 2003b; Wang et al., 2010b).

Furthermore, ICC have been observed to contract rhythmically, and while the functional significance of this is currently unclear, Thuneberg suggested that contractile activity in ICC may play a role in sensing stretch (Thuneberg and Peters, 2001; Huizinga et al., 2010).

### 2.2.6 Clinical Significance of ICC

Since the recognition that ICC play a critical role in normal GI motility, pathophysiological associations between ICC and motility disorders have been reported for all regions of the gut (Farrugia, 2008; Huizinga et al., 2009). Interpreting the functional significance of
defects in ICC is complicated by the fact that other cell types, including enteric nerves, are also affected in many of these conditions. In particular, there is a strong association between loss of ICC and loss of enteric nerves in motility diseases. Further confounding the issue is the large variety of techniques used to identify ICC in human tissue and the quality of the tissue used (Garrity et al., 2009; Knowles et al., 2009). Most of the work done on normal tissue has used different fixation and visualisation techniques from those used in pathological states, making comparisons difficult. Additionally, human ICC numbers vary by site and decline markedly with age, making use of proper controls vital (Gomez-Pinilla et al., 2011).

Despite these issues, associations of likely functional significance are now established in several disorders, notably in diabetic and idiopathic gastroparesis or delayed gastric emptying (Grover et al., 2011; Kashyap and Farrugia, 2010) and slow transit constipation (He et al., 2000), in both of which ICC network depletion is now regarded as a pathological hallmark. ICC defects have also been implicated in intestinal pseudo-obstruction (Isozaki et al., 1997), inflammatory conditions (Der et al., 2000), ileus or impaired motility (Zhou et al., 2011), and bowel obstruction (Chang et al., 2001). An absolute loss of ICC is not required to produce a functional defect (Ordög et al., 2002). Because ICC act as a network to generate and propagate signals, abnormal function or patchy loss can have significant effects even if total numbers are not markedly affected (Du et al., 2010c), although functional defects may not arise until changes in ICC networks cross a threshold (Gomez-Pinilla et al., 2011; Tharayil et al., 2010).

A developing area of clinical interest is ICC ion channelopathies, which have been implicated in irritable bowel syndrome. In a survey of patients with a mutation in the SCN5A gene, which encodes a Na\(^+\) channel in ICC, 50% had abdominal pain and 65% had a GI symptom complex (Locke et al., 2006). Investigation of 49 subjects who had been diagnosed with irritable bowel syndrome found that one patient had a loss-of-function mutation in SCN5A, resulting in a decrease in whole-cell Na\(^+\) current, delayed channel activation and decreased mechanosensitivity (Saito et al., 2009).
2.2.7 Unravelling the Mechanism of Slow Wave Generation

Slow wave depolarisations recorded from ICC-MY are often called pacemaker potentials or driving potentials in recognition of their role in initiating slow waves, and recordings from ICC-CM have been called regenerative potentials (Dickens et al., 1999; Suzuki and Hirst, 1999). This terminology can be used to differentiate between membrane potentials recorded directly from ICC and those recorded from SMC, which differ in their amplitudes and constituent ion currents, so the term ‘pacemaker potential’ is preferentially used in this thesis when referring to electrical activity recorded from ICC-MY. Nevertheless, pacemaker potentials, regenerative potentials and SMC slow waves all have the same electrical origins in ICC, and the term ‘slow wave’ is often used to refer to electrical pacemaker activity recorded from all cell types in the GI tract, particularly in the modelling field (Corrias and Buist, 2008; Faville et al., 2009; Sanders et al., 2006).

The discovery that ICC were the GI pacemakers gave rise to intense interest in the mechanism by which ICC generate and propagate slow waves. The ICC pacemaker mechanism involves intracellular Ca\(^{2+}\) handling and activation of Ca\(^{2+}\)-dependent ion currents. The most elaborate theory regarding ICC function was proposed by Sanders et al. (2006) following a series of publications investigating slow waves primarily using cultured ICC (Kim et al., 2002; Koh et al., 2002; Ward et al., 2000). Reservations about the effects of culture on the integrity of the ICC pacemaker mechanism (Epperson et al., 2000; Zhu et al., 2009) and the discovery of a novel candidate pacemaker channel in ICC (Gomez-Pinilla et al., 2009; Hwang et al., 2009) necessitated rethinking how ICC produce pacemaker activity. Therefore, the remainder of this chapter gives a brief introduction to the methods used to investigate slow wave activity (Section 2.3), then reviews the Ca\(^{2+}\) handling mechanisms (Sections 2.4–2.5) and ion channels (Section 2.6) that have been identified in ICC and considers how these components may combine to generate slow waves (Sections 2.7–2.9).
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2.3 Methods for Investigating Gastrointestinal Electrical Activity

The fundamental element of cellular electrical activity is the ion channel. Ion channels in the plasma membrane and in the membranes of cell organelles coordinate in setting the RMP and responding to a variety of mechanical, chemical and electrical stimuli. In electrically active cells like ICC and SMC, specialised ion channels generate, amplify and propagate electrical signals. Experimental tools have been developed to study the components of the GI electrical system from the single ion channel to the organ level, each with distinct benefits and drawbacks. The electrical activity of ICC and GI tissue can be studied using the electrode recording techniques described below, with several electrophysiological, molecular and pharmacological tools available to categorise and identify the ion currents being recorded.

2.3.1 Recording Electrical Activity

At least two electrodes are required to study bioelectrical activity: a recording electrode and a reference electrode. The voltage difference between these electrodes is the measured potential. Cellular recordings are typically performed in either current clamp or voltage clamp mode, and the design and use of the recording electrode depends on the sample of interest.

The activity of single ion channels or small populations of channels in isolated cells can be studied using patch-clamp. In a patch-clamp setup a small area of cell membrane is aspirated into a glass micropipette recording electrode filled with an electrolyte solution, forming a tight seal between the membrane and glass, which enables currents through single ion channels to be recorded (Sakmann and Neher, 1995). This typically requires dissociation of cells from tissue, or expression of the ion channels of interest in heterologous cell lines like human embryonic kidney (HEK) 293 cells, but in situ patch clamp recording is possible under certain conditions, and has been demonstrated in ICC (Wang et al., 2008a).
To study the entire population of ion channels in a cell, whole-cell patch-clamp may be employed (Strege et al., 2003b). In this case, the seal is formed as above and then the membrane patch is ruptured so the electrode solution becomes continuous with the cell interior. In this mode of recording, single channels generally cannot be studied. The benefits of whole-cell recording include the ability to exchange the intracellular and extracellular solutions, and to study the effect of drug application on different ion currents, although unintended replacement of the intracellular solution with the electrode solution is also a drawback of whole-cell patch-clamp (Liem et al., 1995). The exchange of intracellular and electrode solutions during whole-cell patch-clamp can be reduced by creating a perforated patch using pore-forming antibiotics instead of completely rupturing the membrane within the patch (Lippiat, 2008).

If the electrical activity of intact tissue is of interest then sharp microelectrode intracellular recording or extracellular electrodes may be employed (Dickens et al., 1999). Intracellular recording lacks the ability to record currents under voltage clamp, but a significant advantage is that the complex autonomous electrical activity of different cell types can be studied in their near-native environment. However, as with whole-cell patch-clamp, the microelectrode filling solution can leak into the cell and alter intracellular ion concentrations (Fromm and Schultz, 1981).

Extracellular recordings, typically performed using surface contact electrodes, are a valuable recording technique for in vivo studies. Extracellular signals are generated by transmembrane currents arising due to the voltage gradient between excited and resting tissues, and correspond to the weighted summation of activity from a localised region of tissue around the electrode (Angeli et al., 2013). Detailed spatiotemporal maps can be generated from multi-electrode recordings, describing slow wave propagation sequences in high-resolution (Lammers et al., 2005; O’Grady et al., 2010).

2.3.2 Model Tissues

In the quest to understand the molecular mechanisms of GI electrical activity, the functional details and arrangement of the ion channels and associated proteins needs to be understood.
Opportunities to study human GI physiology are limited (Lee et al., 2007b; Strege et al., 2003b), so multiple animal models have been employed, including mice (Gibbons et al., 2009; Kito and Suzuki, 2003; Lowie et al., 2011), guinea-pigs (Dickens et al., 1999; Kito et al., 2002b), dogs (Ward et al., 2004), and rats (Wang et al., 2008a).

The study of electrical activity arising specifically from ICC can be facilitated by isolating the ICC from the surrounding smooth muscle tissue. However, freshly dissociated ICC are difficult to identify amongst the significantly more numerous SMC (Koh et al., 1998; Strege et al., 2003b) so cultured ICC have often been used preferentially, despite difficulties associated with studying ICC in culture. Cell culture is known to cause substantial changes in cell behaviour, such as rearrangement of independent ER Ca\textsuperscript{2+} stores in canine pulmonary artery SMC into a common Ca\textsuperscript{2+} store, thereby altering the effects of Ca\textsuperscript{2+} store-depletion (Ng et al., 2008). ICC in culture quickly change phenotype, losing their automaticity and becoming more like SMC (Epperson et al., 2000; Zhu et al., 2009). The alteration of cell structure and function must be kept in mind when interpreting diverse experimental results from cultured and freshly dispersed ICC.

In an effort to overcome some of these problems, a new mouse model has been developed with ICC expressing green fluorescent protein (GFP)-Kit (Ro et al., 2010), which makes identification of freshly dispersed ICC easier (Zhu et al., 2009). Nevertheless, work performed in ex vivo biological systems requires great effort and patience.

### 2.3.3 Identifying Ion Channels

Ion channels can be identified using an electrophysiological approach by employing patch- or whole-cell clamp, or using molecular biology by amplifying genes or their products from tissues or single cells. In electrophysiology, electrical signals are frequently composites of multiple ion currents. Identification of the contributing channels requires a decomposition of the current into individual components by defining properties of the channels such as ion selectivity, gating stimuli, and sensitivity to pharmacological agents.

Once candidate ion channels and modulating proteins are identified, these proteins can be expressed in heterologous cell systems, such as HEK 293 cells, Chinese hamster ovary
(CHO) cells or *Xenopus* oocytes, so that electrophysiology of the protein can be studied in detail down to the single channel level. One way to classify ion channels is by gating sensitivity to voltage, ligands, ions, or mechanical stimuli. These channels gate from a closed to an open state over a narrow range of the specific stimulus, and gating sensitivity may often be described by a simplified Boltzmann equation (Dubois et al., 2009):

\[
\frac{g}{g_{\text{max}}} = \frac{1}{1 + \exp\left(\frac{(V_m - V_h)}{-k}\right)},
\]

(2.1)

where for a voltage-gated channel, \(g\) is the steady-state conductance of a population of channels, \(g_{\text{max}}\) is maximal conductance, \(V_m\) is membrane potential, \(V_h\) is the membrane potential at which \(g\) is half-maximal, and \(k\) is the slope factor. \(k\) is equal to \(zF/RT\), where \(z\) is the valence of the gating charge for the channel; \(F\) is the Faraday constant, 96 485 C mol\(^{-1}\); \(R\) is the gas constant, 8.314 J mol\(^{-1}\) K\(^{-1}\); and \(T\) is absolute temperature in kelvins.

Ion channels are also classified by their ion selectivity—anion, non-selective cation (NSC), Ca\(^{2+}\), Cl\(^{-}\), potassium (K\(^{+}\)), Na\(^{+}\), etc. Candidate ions can be identified by the reversal potential of a particular ion current, equal to the Nernst equilibrium potential,

\[
E_S = \frac{RT}{zSF} \ln \frac{[S]_o}{[S]_i},
\]

(2.2)

where \(E_S\) is the equilibrium potential for the ionic species \(S\); \(z_S\) is the valence of ionic species \(S\) (+1 for Na\(^{+}\) or K\(^{+}\), +2 for Ca\(^{2+}\), or −1 for Cl\(^{-}\)); and \([S]_o\) and \([S]_i\) are the extracellular and intracellular concentrations of ionic species \(S\), respectively.

Individual ions can then be eliminated from the solutions, and subsequent loss of current indicates selectivity for the eliminated ion, which is significant progress toward ion channel identification. Additional functional description of ion channels may use pharmacological modification.
2.3.4 Drugs and their Drawbacks

A key hindrance in experimental studies investigating ICC pacemaker mechanisms has been the lack of specificity of many of the pharmacological agents used to identify membrane ion currents and intracellular ion transfer mechanisms. Meaningful discussion about experimental results must be framed by a good understanding of this limitation. The main agents employed, their intended targets and other effects are as follows:

2-aminoethoxydiphenyl borate (2-APB) has been used in ICC as an inhibitor of inositol 1,4,5-trisphosphate (IP$_3$)-mediated Ca$^{2+}$ release from the ER. However, 2-APB reliably inhibits store-operated calcium entry (SOCE)$^3$ through both transient receptor potential (TRP) channels (Bootman et al., 2002), which tend to be non-selectively permeable to cations, and through highly Ca$^{2+}$-selective calcium release activated calcium (CRAC) channels. 2-APB has varying effects on CRAC channels, but in general > 10µM 2-APB inhibits CRAC channels, while low micromolar concentrations of 2-APB potentiate CRAC channels (Peinelt et al., 2008; Prakriya and Lewis, 2001; Smyth et al., 2010). At similar concentrations, 2-APB inhibits a variety of store-independent TRPC (canonical TRP) and TRPM (melastatin TRP) channels (Bootman et al., 2002; Kim et al., 2005; Xu et al., 2005). IP$_3$ receptors (IP$_3$Rs) are inhibited by 2-APB with an IC$_{50}$ of 42µM–1 mM, depending on IP$_3$ concentration (Bootman et al., 2002; Maruyama et al., 1997). 2-APB has also been shown to reversibly block gap junction coupling (Bai et al., 2006; Harks et al., 2003), and to inhibit intracellular Ca$^{2+}$ transporters including sarco-endoplasmic reticulum calcium ATPase (SERCA) pumps (Bootman et al., 2002) and mitochondrial Ca$^{2+}$ efflux via the mitochondrial sodium/calcium exchanger (mNCX) (Bootman et al., 2002; Prakriya and Lewis, 2001).

Xestospongin C blocks IP$_3$R-mediated Ca$^{2+}$ release from the ER, but can also inhibit SOCE (Bootman et al., 2002) and SERCA pumps (De Smet et al., 1999; Solovyova et al., 2002).

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$^3$See Section 2.5 for more information on store-operated Ca$^{2+}$ entry.
2.3. Methods for Investigating Gastrointestinal Electrical Activity

**Caffeine and heparin** both inhibit IP$_3$Rs and activate ryanodine receptors (Ehrlich et al., 1994). Caffeine also inhibited Ca$^{2+}$-inhibited NSC currents in ICC (Jin et al., 2009).

**SK&F 96365** was originally shown to inhibit receptor-mediated Ca$^{2+}$ entry with an IC$_{50}$ around 10µM (Merritt et al., 1990), but since then it has been commonly used to inhibit store-operated CRAC channels (Franzius et al., 1994; Várnai et al., 2009; Touchberry et al., 2011), and non-selective TRPC (Lin et al., 2004; Torihashi et al., 2002) and TRPM7 channels (Kim et al., 2005). SK&F 96365 has also been shown to inhibit voltage-gated L-type and T-type Ca$^{2+}$ channels at similar concentrations (Merritt et al., 1990; Singh et al., 2010). It has a bevy of other responses, especially at concentrations above 100µM, and is widely regarded as a non-specific agent (Jan et al., 1999; Schwarz et al., 1994; Singh et al., 2010).

**Nickel ions** (Ni$^{2+}$) are typically used to block T-type Ca$^{2+}$ channels, but the concentration of Ni$^{2+}$ required depends on the T-type channel isoform. Ca$_{\text{V3.2}}$ T-type channels are inhibited by 10µM to 50µM Ni$^{2+}$, whereas Ca$_{\text{V3.1}}$ and Ca$_{\text{V3.3}}$ channels require 10- to 20-fold greater Ni$^{2+}$ concentration (Lee et al., 1999; Perez-Reyes, 2003). Ni$^{2+}$ in the same concentration range of 10µM to 2mM inhibits store-operated NSC currents through TRP channels (Flemming et al., 2003; Ng and Gurney, 2001; Ng et al., 2009; Trepakova et al., 2001). In addition, Ni$^{2+}$ inhibited receptor-mediated Ca$^{2+}$-permeant NSC channels at Ni$^{2+}$ concentrations of at least 50µM (Inoue, 1991; Lotshaw and Sheehan, 1999), and inhibited the plasma membrane sodium/calcium exchanger (NCX) in cardiac myocytes with a dissociation constant ($K_d$) of 0.16mM to 0.29mM (Hinde et al., 1999).

**Mibefradil** inhibits T-type Ca$^{2+}$ channels when used in concentrations greater than 0.1µM, but it also inhibits Na$_{\text{V1.5}}$ sodium channels at 0.3µM, and L-type Ca$^{2+}$ channels at 1µM (Strege et al., 2005). It has been shown to inhibit voltage-dependent K$^+$ currents in vascular SMC with an apparent $K_d$ of 1.08µM (Hong et al., 2012).

**Cl$^-$ channel inhibitors:** A large number of pharmacological agents are known block-
ers of Cl− channels, including fenamates: niflumic acid and flufenamic acid; stilbenes: 4,4’-diisothiocyanatostilbene-2,2’-disulphonic acid (DIDS) and 4-acetamido-4-isothiocyanatostilbene-2,2’-disulphonic acid (SITS); and 9-anthracene carboxylic acid (9-AC) (Dick et al., 1999; Park et al., 2005; White and Aylwin, 1990). However, these drugs have of course been shown to inhibit other channels. Niflumic acid, DIDS, and 9-AC inhibited NSC currents in ICC (Koh et al., 2002; Takeda et al., 2008; Walker et al., 2002). NSC currents were also inhibited by niflumic acid and flufenamic acid in pancreatic cells (Gögelein et al., 1990) and by DIDS in vascular SMC (Welsh et al., 2000).

L-type Ca2+ channels in vascular and colonic SMC were inhibited by flufenamic acid, DIDS, and 9-AC (Dick et al., 1999; Doughty et al., 1998). Niflumic acid, DIDS, and 9-AC caused ER Ca2+ release in vascular SMC (Cruickshank et al., 2003).

Fenamates reportedly stimulate delayed rectifier K+ currents (Busch et al., 1994; Farrugia et al., 1993) and BK Ca2+-activated K+ channels in vascular myocytes (Greenwood and Large, 1995; Hogg et al., 1994; Ottolia and Toro, 1994). DIDS also stimulated human delayed rectifier K+ channels expressed in Xenopus oocytes (Busch et al., 1994).

In addition, niflumic acid can act as an agonist to Ca2+-activated Cl− currents under the conditions of negative membrane potential and high intracellular Ca2+ concentration ([Ca2+]i; 250 and 500 nM), and washout of niflumic acid can enhance Ca2+-activated Cl− currents (Piper et al., 2002). Physiologically, ICC always have negative membrane potential, and it is likely that Ca2+ transients reach higher than 250 nM (Means and Sneyd, 2010).

### 2.4 Calcium Oscillations in ICC

The importance of Ca2+ dynamics in the ICC pacemaker mechanism was well established based on early work in the ICC field, which typically involved inhibiting a component speculated to partake in the pacemaker mechanism in order to observe the effects on
aspects of pacemaker activity including Ca\(^{2+}\) oscillations, spontaneous inward currents, pacemaker potentials, regenerative potentials, and slow waves.

Despite obvious differences between pacemaker potentials from the stomach and those from the small intestine, notably the lower frequency and longer duration in the stomach, the mechanism by which pacemaker potentials are generated appears to be much the same in all types of pacemaker ICC. These discoveries will be summarised here, and the interested reader is directed to a comprehensive review by Sanders et al. (2006) for more information. When considering these experimental results it is important to keep in mind that many of the pharmacological agents employed have been shown to have non-specific effects, as detailed in Section 2.3.4.

### 2.4.1 Endoplasmic Reticulum

Uptake and release of Ca\(^{2+}\) from internal stores in the ER is a key dynamic in ICC pacemaker activity, and has long been thought to set the pace of the intracellular biochemical clock (Liu et al., 1995). Cell-wide Ca\(^{2+}\) oscillations in ICC are likely to be similar in magnitude to global Ca\(^{2+}\) transients in cardiac myocytes, in which \([\text{Ca}^{2+}]_i\) increases from a resting level around 100 nM to a peak around 1 \(\mu\)M (Best and Kamp, 2012; Shaw and Colecraft, 2013), although localised Ca\(^{2+}\) transients in ICC are likely to be many times larger than this (Means and Sneyd, 2010).

The importance of ER Ca\(^{2+}\) stores is evidenced by the inhibition of all types of pacemaker activity—including pacemaker potentials, slow waves, pacemaker currents, and intracellular Ca\(^{2+}\) oscillations—when uptake of Ca\(^{2+}\) via the SERCA is blocked by cyclopiazonic acid (CPA) or thapsigargin (Lee et al., 2007b; Liu et al., 1995; Malysz et al., 2001; Suzuki and Hirst, 1999; Ward et al., 2000).

Pharmacological evidence suggests that IP\(_3\)Rs are important for pacemaking, because drugs such as caffeine, xestospongin C, heparin, and 2-APB inhibit pacemaker activity, either by abolishing pacemaker activity completely or by reducing the frequency, amplitude and duration of pacemaker activity (Hirst and Edwards, 2001; Lee et al., 2007b; Liu et al., 1995, 2005a; Malysz et al., 2001). The non-specificity of many IP\(_3\)R inhibitors—especially 2-
APB (Bootman et al., 2002), but also xestospongins C (De Smet et al., 1999; Solovyova et al., 2002) and caffeine (Jin et al., 2009)—means that the results from experiments using these pharmacological agents need to be interpreted with caution. Nevertheless, well-designed pharmacological experiments can be useful. The simultaneous application of carbachol and either CPA or thapsigargin resulted in an initial increase in slow wave frequency due to stimulation of IP$_3$Rs followed by a rapid decline in frequency due to ER depletion. Similar effects were observed in experiments where carbachol was replaced by other compounds that activate IP$_3$ production via phospholipase C. In addition, sensitising IP$_3$Rs with thimerosal caused an increase in slow wave frequency, while inhibiting phospholipase C caused a decrease in slow wave frequency. These results indicate that IP$_3$-mediated ER Ca$^{2+}$ release is essential for initiating and setting the timing of pacemaker activity (Lowie et al., 2011; Malysz et al., 2001).

The strongest evidence for the role of IP$_3$Rs is the lack of pacemaker activity in gastric smooth muscle tissue from mutant mice lacking type 1 IP$_3$R (IP$_3$R1) (Suzuki et al., 2000). IP$_3$R1 has been identified in ICC from mouse stomach and small intestine (Chen et al., 2007; Liu et al., 2005a; Lowie et al., 2011) and is likely to be the primary candidate for Ca$^{2+}$ release in the pacemaker cycle, but type 2 IP$_3$R (IP$_3$R2) has also been found in mouse gastric and small intestinal ICC (Aoyama et al., 2004; Liu et al., 2005a).

The majority of studies on ICC have shown that ryanodine receptors on the ER do not play a crucial role in ICC pacemaker activity (Lee et al., 2007b; Malysz et al., 2001; van Helden et al., 2000; Ward et al., 2000). Type 3 ryanodine receptors are expressed in murine ICC and some studies found that ryanodine can inhibit Ca$^{2+}$ cycling but does not affect pacemaker currents (Aoyama et al., 2004; Liu et al., 2005b; Wang et al., 2010c).

### 2.4.2 Mitochondria

Mitochondria may play a role in ICC pacemaking by taking up Ca$^{2+}$ via mitochondrial uniporters and releasing Ca$^{2+}$ via mNCX. Mitochondrial uncouplers (FCCP and CCCP), and respiratory chain inhibitors (antimycin and rotenone) were found to abolish pacemaker activity or reduce frequency, amplitude and propagation in various tissues, including
human small intestine (Lee et al., 2007b), mouse small intestine (Park et al., 2006; Ward et al., 2000), mouse colon (Yoneda et al., 2002), canine stomach (Ward et al., 2004), canine colon (Ward et al., 2003), and guinea-pig stomach (Kito et al., 2002a). These effects did not appear to be due to a reduction in adenosine triphosphate (ATP) availability because oligomycin, which specifically disrupts mitochondrial ATP production, did not have any affect on slow waves or pacemaker potentials (Ward et al., 2003; Yoneda et al., 2002).

Inhibiting the mitochondrial Ca$^{2+}$ uniporter abolished spontaneous currents in cultured mouse small intestine ICC (Ward et al., 2000), suggesting that mitochondrial Ca$^{2+}$ uptake is important for pacemaking. Inhibiting the mNCX with clonazepam or CGP-37157—which may also inhibit SERCA (Neumann et al., 2011) and L-type Ca$^{2+}$ channels (Chalmers and McCarron, 2009)—was found to inhibit pacemaker currents and pacemaker potentials in cultured ICC from mouse small intestine (Kim et al., 2006a), but in ICC-MY \textit{in situ} the effect of CGP-37157 on Ca$^{2+}$ oscillations was weak and variable (Lowie et al., 2011), indicating that the importance of mitochondria in mediating Ca$^{2+}$ oscillations in ICC may be exaggerated in culture. Nevertheless, the lack of inhibition by CGP-37157 in ICC-MY \textit{in situ} does not rule out a possible role for mitochondria in intracellular Ca$^{2+}$ handling.

In guinea-pig colon SMC it is mitochondrial Ca$^{2+}$ uptake, rather than efflux, that plays a crucial role in maintaining IP$_3$R-mediated intracellular Ca$^{2+}$ release (Chalmers and McCarron, 2009).

### 2.4.3 Setting the Clock

IP$_3$R-mediated Ca$^{2+}$ release is clearly involved in the initiation of pacemaker activity, but the mechanism by which the frequency of the pacemaker cycle is set is ill-defined. Lowie et al. (2011) suggested two possible mechanisms for setting the timing of pacemaker activity via an internal clock: activation and delayed inhibition of IP$_3$Rs by intracellular Ca$^{2+}$; or oscillations in IP$_3$ concentration ([IP$_3$]), perhaps controlled by Ca$^{2+}$ feedback on IP$_3$ synthesis or breakdown. Rhythmically activated ion channels or transporters were also mentioned as a possible alternative to an intracellular clock, although the continuation of Ca$^{2+}$-dependent rhythmic pacemaker currents in the absence of rhythmic depolarisations
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during voltage clamp (Koh et al., 2002; Thomsen et al., 1998; Tokutomi et al., 1995) indicates that the pacemaker clock is independent of voltage-dependent ion currents.

2.5 Store-Operated Calcium Entry

It is clear that Ca\(^{2+}\) cycling between the ER and cytosol is a key part of the ICC pacemaker mechanism. The classical view of Ca\(^{2+}\) oscillations in ICC is that changes in [Ca\(^{2+}\)]\(_i\), directly activate Ca\(^{2+}\)-dependent ion channels in the plasma membrane to drive depolarisation, either Ca\(^{2+}\)-activated channels—e.g., Cl\(^-\) channels (Hirst et al., 2002a; Kito et al., 2002a; Kito and Suzuki, 2003; Wright et al., 2012) or NSC channels (Goto et al., 2004; Lees-Green et al., 2011b; Takeda et al., 2008)—or Ca\(^{2+}\)-inhibited non-selective cation channels (Koh et al., 2002; Takeda et al., 2008). However, Ca\(^{2+}\) store depletion may also play a part in ICC pacemaking (Daniel et al., 2009; Liu et al., 2005a; Torihashi et al., 2002). Store-operated calcium entry (SOCE) is the activation of Ca\(^{2+}\)-permeable ion channels—so-called store-operated Ca\(^{2+}\) (SOC) channels—in the plasma membrane in response to depletion of Ca\(^{2+}\) stores in the ER.

There is a wealth of information about SOCE, and many review articles covering the topic in detail are available (Smyth et al., 2010; Stiber and Rosenberg, 2011). Here, a brief overview of SOCE is provided, with a particular focus on the aspects most relevant to the consideration of a possible role for SOC channels in ICC pacemaking.

2.5.1 Characteristics of SOCE

SOCE—also known as capacitative calcium entry (CCE)—occurs when the Ca\(^{2+}\) stores within the ER are depleted. Experimentally, ER stores can be depleted by activating IP\(_3\)R-mediated Ca\(^{2+}\) release while using thapsigargin or CPA to inhibit reuptake of Ca\(^{2+}\) by SERCA pumps. Using this experimental protocol, an initial Ca\(^{2+}\) transient is typically observed, attributed to ER Ca\(^{2+}\) release, followed by a sustained increase in [Ca\(^{2+}\)]\(_i\), attributed to Ca\(^{2+}\) influx via SOC channels.

The first SOC channel to be characterised was the Ca\(^{2+}\) release activated Ca\(^{2+}\) (CRAC) channel. CRAC channels have high Ca\(^{2+}\) selectivity and very small single channel
conductance—as small as 20 fS—that can make native CRAC currents difficult to detect (Parekh and Putney, 2005; Smyth et al., 2010). Non-CRAC store-operated currents have also been observed; these are typically non-selective cations currents and are likely to be carried by TRPC channels (Ng et al., 2009; Salido et al., 2009; Yuan et al., 2012).

### 2.5.2 Molecular Nature of SOCE

SOCE and CRAC channels have been studied extensively since Putney (1986) first proposed CCE but the molecular identities of the channels involved in SOCE have only been discovered within the last decade.

Liou et al. (2005) and Roos et al. (2005) identified stromal interaction molecule (STIM)1 and STIM2 as the Ca\textsuperscript{2+} sensors in the ER; much of the subsequent research has focused on STIM1 as the primary Ca\textsuperscript{2+} sensor. STIM1 is localised in the ER with a Ca\textsuperscript{2+} binding domain in the ER lumen, and forms clusters near the plasma membrane during ER Ca\textsuperscript{2+} depletion (Liou et al., 2005; Zhang et al., 2005). The clustering of STIM1 occurs because dissociation of Ca\textsuperscript{2+} from STIM1 upon ER store depletion causes destabilisation of the protein structure, resulting in oligomerisation of STIM1 and subsequent translocation of STIM1 towards the plasma membrane (Liou et al., 2007; Stathopulos et al., 2006, 2008). Each STIM1 molecule binds one Ca\textsuperscript{2+} ion with an apparent $K_d$ around 200 to 250µM, although one estimate placed the STIM1 $K_d$ as high as 600µM (Stathopulos et al., 2006, 2008). Similarly, the EC\textsubscript{50} for the Ca\textsuperscript{2+} dependence of the STIM1 translocation process (i.e., Ca\textsuperscript{2+} dissociation, STIM1 oligomerisation, and translocation to the plasma membrane) was estimated to be 210µM (Brandman et al., 2007).

STIM2 is also associated with SOCE but its exact role is unclear (Hoth and Niemeyer, 2013). In different studies STIM2 has been identified as either necessary (Liou et al., 2005) or not important for SOCE (Roos et al., 2005). STIM2 has also been shown to mediate store-independent activation of CRAC channels (Parvez et al., 2008) and to inhibit STIM1-mediated SOCE (Soboloff et al., 2006a). The $K_d$ for Ca\textsuperscript{2+} dissociation from STIM2 was estimated to be around 500µM (Zheng et al., 2008), and the EC\textsubscript{50} for Ca\textsuperscript{2+} dependence of the STIM2 translocation process was around 400µM, making STIM2
less sensitive than STIM1 to Ca\(^{2+}\) (Brandman et al., 2007). On the other hand, the Ca\(^{2+}\) affinity of STIM2 is close to the resting ER Ca\(^{2+}\) concentration (estimated to be approximately 400\(\mu\)M), so STIM2 can respond to partial ER depletions and may regulate basal ER Ca\(^{2+}\) (Brandman et al., 2007).

Shortly after the discovery of STIM1 as the ER Ca\(^{2+}\) sensor, a novel protein was discovered to be a crucial component of CRAC channel-mediated Ca\(^{2+}\) influx (Feske et al., 2006; Vig et al., 2006b; Zhang et al., 2006). Feske et al. (2006) named this protein Orai1 after the ‘Orai’ (literally the ‘Hours’) who were keepers of the gates of heaven in Greek mythology (Stewart, 2005). Subsequently, interaction between STIM1 and Orai1 was shown to be important for mediating CRAC currents (Peinelt et al., 2006). Coexpression of STIM1 and Orai1 was shown to reconstitute SOC channel function; Orai2 and Orai3 could also produce SOCE when coexpressed with STIM1 but with less efficacy than Orai1, whereas coexpression of STIM2 and Orai1 caused store-independent Ca\(^{2+}\) entry (Soboloff et al., 2006b; Mercer et al., 2006).

Orai proteins were found to form the Ca\(^{2+}\)-selective pore of the CRAC channel (Prakriya et al., 2006; Yeromin et al., 2006; Vig et al., 2006a). STIM1 does not incorporate into the plasma membrane, but binds with oligomeric assemblies of Orai1 at sites of close apposition between the plasma membrane and the Ca\(^{2+}\) store membrane (Mercer et al., 2006; Vig et al., 2006a). Both Orai1 and Orai3 form dimers under resting conditions (Demuro et al., 2011) and STIM1 induces dimerisation of Orai dimers (Penna et al., 2008); as a result four Orai1 subunits make up the CRAC channel pore in association with two STIM1 molecules (Ji et al., 2008; Mignen et al., 2008). More recently, the Drosophila Orai homologue was shown to be hexameric in CRAC channels (Hou et al., 2012). When both tetrameric and hexameric assemblies of human Orai1 were expressed in HEK 293 cells, the tetrameric channel had the properties of native CRAC channels—including high Ca\(^{2+}\)-selectivity—whereas the hexameric channel appeared to be a store-operated NSC channel (Thompson and Shuttleworth, 2013).

Orai2 and Orai3 also form SOC channels but their properties differ from those of Orai1 and their physiological roles have yet to be established (Hoth and Niemeyer, 2013; Lis et al., 2007). Orai1 can form heteromultimers with Orai2 and Orai3 to produce CRAC
channels with altered functional characteristics, enabling the Ca\(^{2+}\) signal generated by SOCE to be modulated by alternative oligomerisation (Lis et al., 2007).

While oligomerisation of Orai1 and STIM1 forms the CRAC channel, TRPC channels can also interact with STIM to facilitate store-operated calcium entry (Yuan et al., 2012). For example, SOCE in mouse vascular SMC is mediated by STIM1 activating and co-immunoprecipitating with TRPC1 (Ng et al., 2009). Furthermore, in some cases both Orai and TRPC have been shown to contribute to SOCE. Colocalisation of both TRPC1 with STIM1 and Orai1 with STIM1 was observed in hypoxic vascular SMC, and all three proteins appeared to be important for SOCE (Ng et al., 2012). Native SOCE in HEK 293 cells required co-expression of Orai1 and TRPC1 and the functioning of both proteins as channels was essential (Kim et al., 2009b). TRPC3 and TRPC6 overexpressed in HEK 293 cells also acted as STIM1-mediated store-operated channels upon expression of Orai1, Orai2 or Orai3 (Liao et al., 2007). Conversely, TRPC channels mediate store-independent currents in other cell systems, including receptor-operated channels (Zarayskiy et al., 2007). Store-operated and receptor-operated mechanisms may also act in combination. IP\(_3\)Rs coupled to TRPC channels are hypothesised to block STIM1 from binding to TRPC; in this model, IP\(_3\) causes dissociation of IP\(_3\)Rs from TRPC, allowing STIM1 to bind and form a SOC channel (Yuan et al., 2012).

### 2.5.3 A Possible Role for SOCE in ICC

In ICC cultured from mouse small intestine, Torihashi et al. (2002) demonstrated the occurrence of SOCE following Ca\(^{2+}\) store depletion in ICC by inhibiting SERCA pumps with thapsigargin. In addition, the SOC and TRP channel inhibitor SK&F 96365 and Ca\(^{2+}\)-free external solution both inhibited Ca\(^{2+}\) oscillations (Torihashi et al., 2002). SK&F 96365 also inhibited spontaneous Ca\(^{2+}\) oscillations in ICC cultured from mouse stomach, as did 10\(\mu\)M 2-APB (Liu et al., 2005a). Daniel et al. (2009) proposed that store-operated channels are important for maintaining the pacemaker frequency in ICC.

Further evidence for the importance of SOCE in ICC comes from the inhibitory effects of 2-APB on pacemaker activity, typically reducing the frequency and amplitude of Ca\(^{2+}\)
transients or abolishing pacemaker activity entirely (Liu et al., 2005a; Lowie et al., 2011; Park et al., 2006). The ICC literature generally describes 2-APB as an IP₃R inhibitor, but 2-APB actually has complex effects on Ca²⁺ influx and release, as described in Section 2.3.4. The effects of 2-APB on ICC must be interpreted with caution, but 2-APB appears to be a more consistent and potent inhibitor of SOC channels than IP₃Rs, suggesting a role for SOCE in ICC pacemaking.

The identity of the SOC channel in ICC is unclear. Torihashi et al. (2002) suggested that TRPC4 channels may be store-operated channels in ICC, although TRPC4 channels do not play an essential role in pacemaking as TRPC4⁻/⁻ mice display normal electrical activity (Kim et al., 2006b; Sanders et al., 2006). A number of other TRPC and TRPM channels have been identified in ICC (Chen et al., 2007; Epperson et al., 2000; Liu et al., 2005a), and Daniel et al. (2009) reported the presence of Orai2 in mouse small intestine smooth muscle tissue. Both Daniel et al. (2009) and Torihashi et al. (2002) speculated that SOC channels are associated with caveolae in ICC.

Store depletion and refill cycles in non-excitatory cells typically occur on slow time-scales, on the order of tens of seconds, due to the time STIM1 takes to cluster and translocate to the plasma membrane (Edwards et al., 2010), whereas pacemaker activity in mouse small intestine ICC can have a period less than 2 s (Thomsen et al., 1998). Conversely, rapid SOCE has been observed in skeletal muscle cells, in which a splice variant of STIM1 permanently localised at the plasma membrane can activate SOC channels within milliseconds of depletion occurring (Darbellay et al., 2011; Edwards et al., 2010). Therefore, SOCE can function at the frequencies of Ca²⁺ oscillations observed in ICC.

2.6 Ion Channels Found in ICC

Several different types of ion channels are proposed to play an important role in ICC pacemaker mechanisms, including Ca²⁺, Na⁺, Cl⁻, NSC, and K⁺ channels. Each of these channel types are considered in turn, including evidence for their presence in ICC and how they are thought to contribute to the slow wave.
2.6. Ion Channels Found in ICC

2.6.1 Calcium Channels

Two voltage-gated Ca\textsuperscript{2+} channels are found in ICC: the transient, low-voltage-activated T-type channel and the long-lasting, high-voltage-activated L-type channel.

**T-type Ca\textsuperscript{2+} Channel**

A voltage-dependent, dihydropyridine (DHP)-resistant (VDDR) Ca\textsuperscript{2+} current in cultured ICC from murine colon and small intestine was inhibited by the T-type Ca\textsuperscript{2+} channel blockers mibefradil and Ni\textsuperscript{2+} (Kim et al., 2002). Expression of the α\textsubscript{1}H subunit for the Cav\textsubscript{3.2} T-type channel encoded by CACNA1H was confirmed in mouse intestinal ICC (Chen et al., 2007; Gibbons et al., 2009).

Mibefradil, Ni\textsuperscript{2+} and nominally Ca\textsuperscript{2+}-free solution cause a decrease in the upstroke rate of pacemaker potentials in the small intestine and plateau potentials in the colon, suggesting that T-type channels contribute to the upstroke phase (Hotta et al., 2007; Kito and Suzuki, 2003; Kito et al., 2005). Gibbons et al. (2009) showed that the majority of homozygous CACNA1H knockout mice (99\%) died in utero. A surviving CACNA1H knockout mouse produced slow waves with an abnormally slow upstroke rate and frequency, reinforcing the role of T-type Ca\textsuperscript{2+} channels in producing the upstroke phase. T-type current during the upstroke is also believed to contribute to coordinating the plateau phase (Kito et al., 2005; Lee et al., 2007b) and voltage-mediated slow wave propagation (Ward et al., 2004), as discussed below in Section 2.8.

**L-type Ca\textsuperscript{2+} Channel**

L-type Ca\textsuperscript{2+} channels are abundantly expressed in both ICC-MY and ICC-DMP in the mouse small intestine (Chen et al., 2007; Cho and Daniel, 2005), and generate inward current in cultured murine ICC (Kim et al., 2002). L-type channels are blocked by DHPs such as nifedipine and nicardipine. Nifedipine reduced the duration of the plateau potential in murine colon, suggesting L-type current may contribute to the plateau phase in the colon (Hotta et al., 2007; Yoneda et al., 2002). However, DHPs are routinely used to block SMC L-type channels to inhibit contractions in experiments on GI tissue, because
movement can cause artifacts and damage microelectrodes, and earlier studies showed that DHPs do not significantly affect electrical slow waves. DHPs and other L-type channel antagonists caused a small decrease in the amplitude of the slow wave plateau phase in canine gastric muscle (Bayguinov et al., 2007; Ozaki et al., 1991) and reduced $[\text{Ca}^{2+}]_i$ measured from smooth muscle strips but did not affect slow wave production by ICC (Dickens et al., 1999; Suzuki and Hirst, 1999). Therefore, L-type Ca$^{2+}$ channels do not appear to play a significant role in normal slow wave generation. However, when T-type Ca$^{2+}$ channels are inhibited, L-type Ca$^{2+}$ channels can compensate for the loss of the Ca$^{2+}$ influx in order to maintain pacemaker activity (Park et al., 2006).

2.6.2 Sodium Channels

**Na$_V$1.5 Sodium Channel**

SCN5A encodes a voltage-dependent, mechanosensitive sodium channel, Na$_V$1.5, which has been found in human intestinal ICC (Strege et al., 2003b). In intracellular recordings from human jejunum circular muscle, removal of extracellular Na$^+$ or inhibiting Na$_V$1.5 with lidocaine and QX-314 resulted in hyperpolarisation of the RMP, and decreased slow wave upstroke rate and frequency, leading Strege et al. (2003b) to propose that Na$^+$ current contributes to the upstroke phase. Depolarisation causes Na$_V$1.5 current to activate and then rapidly inactivate, which also indicates a role for Na$_V$1.5 current during the upstroke. The Na$_V$1.5 current is mechanosensitive as it was shown to be activated by shear stress (Strege et al., 2003b) and by direct stretch of membrane patches containing Na$_V$1.5 (Beyder et al., 2010).

Expression of Na$_V$1.5 in the gastrointestinal tract is not universal across species. Strege et al. (2007) found Na$_V$1.5 in the circular smooth muscle layer from human and dog jejunum, but not in pig or guinea-pig. Curiously, Na$_V$1.5 was also expressed in mouse circular smooth muscle, but Na$^+$ current was observed in only one out of 21 murine SMC. The expression of SCN5A in ICC from animal models has not yet been investigated.
Background Sodium Channel

A Na\(^+\) leak channel (NALCN) was recently identified in murine small intestine ICC (Kim et al., 2012). NALCN is a voltage-independent cation channel permeable to Na\(^+\), Ca\(^{2+}\) and K\(^+\), and in neurons it carries a background Na\(^+\) current at RMP (Lu et al., 2007). NALCN could help maintain RMP in ICC, but it does not appear to contribute to basal pacemaker activity (Kim et al., 2012).

2.6.3 Chloride Channels

Three types of Cl\(^-\) currents have been observed in ICC: a high-conductance Cl\(^-\) current (Huizinga et al., 2002), a volume-activated Cl\(^-\) current (Park et al., 2005), and a Ca\(^{2+}\)-activated Cl\(^-\) current attributed to the Ano1 channel (Zhu et al., 2009).

Anoctamin 1 Ca\(^{2+}\)-Activated Cl\(^-\) Channel

Ano1 is a Ca\(^{2+}\)-activated Cl\(^-\) channel expressed in human and mouse ICC (Gomez-Pinilla et al., 2009). Previously known as TMEM16A (transmembrane protein 16A), Ano1 was identified by Caputo et al. (2008), Schroeder et al. (2008), and Yang et al. (2008) as a Ca\(^{2+}\)-activated Cl\(^-\) channel with biophysical and pharmacological properties matching the Ca\(^{2+}\)-activated Cl\(^-\) currents that have been characterised in a variety of cell types, including neurons and smooth muscle cells. Yang et al. (2008) named the protein anoctamin 1 because it is an anionic channel that has eight transmembrane domains.

As well as Cl\(^-\), Ano1 is permeable to a number of other monovalent anions, including SCN\(^-\), NO\(_3^-\), I\(^-\), Br\(^-\), F\(^-\), and HCO\(_3^-\) (Jung et al., 2012; Schroeder et al., 2008; Yang et al., 2008). Anoctamins also display cation permeability, and Ano1 appears to be permeable to both Na\(^+\) and K\(^+\) ions (Tian et al., 2012). Ca\(^{2+}\)-activated Cl\(^-\) channels are relatively non-selective, with a P\(_{Cl}:P_{Na}\) of just 10, compared with a P\(_{K}:P_{Na}\) of > 100 for voltage-gated K\(^+\) channels (Hartzell et al., 2005).

The [Ca\(^{2+}\)]\(_i\) required to activate Ano1 depends on membrane potential, and the voltage-current relationship of Ano1 is outwardly rectifying at low (< 1\(\mu\)M) [Ca\(^{2+}\)]\(_i\) (Caputo et al., 2008; Schroeder et al., 2008; Yang et al., 2008). The gating kinetics of Ano1 are described
Zhu et al. (2009) investigated an inward current in ICC that they identified as being carried by Cl\(^-\) ions. The current had a single channel conductance of 7.8 pS, close to the 8.3 pS conductance of Ano1 expressed in HEK 293 cells (Yang et al., 2008). The current activated at \(-72\) mV, but the activation and inactivation kinetics suggested that the current was not directly voltage gated, but rather was dependent on \([Ca^{2+}]_i\) (Zhu et al., 2009).

Ano1 appears to be essential for slow wave production. Ano1 knockout mice have low survival rates, and those that do survive display no slow wave activity (Hwang et al., 2009). The exact role of Ano1 in ICC is not yet clear, but it may generate a pacemaker current. In addition, cholinergic stimulation activated a Ca\(^{2+}\)-activated Cl\(^-\) current in ICC, suggesting Ano1 involvement in neural regulation of ICC pacemaker activity (Zhu et al., 2011).

Ano1 may also contribute in other ways ICC function, because Cl\(^-\) can act as a second-messenger (Orlov and Hamet, 2006). For example, Cl\(^-\) entry through Ano1 facilitates ICC proliferation (Stanich et al., 2011), consistent with studies showing that accumulation of intracellular Cl\(^-\) plays a role in cell growth in a variety of cell types including fibroblasts, vascular and airway SMC, and lymphocytes (Orlov and Hamet, 2006). Nevertheless, quantitative analyses of ICC networks from wild-type and Ano1 knockout mice found no significant differences in the number of ICC or topology of the network structures (Gao et al., 2013; Stanich et al., 2011).

**High-Conductance Chloride Channel**

A high-conductance chloride channel (HCCC) or maxi Cl\(^-\) channel identified in cultured mouse small intestine ICC (Huizinga et al., 2002) is made up of strongly cooperating 30 pS or 60 pS subconductances (Parsons and Sanders, 2008; Wang et al., 2008a; Zhu et al., 2005). The reported properties of this current differ significantly between studies. Huizinga and colleagues reported that the HCCC is Ca\(^{2+}\)-dependent (Huizinga et al., 2002; Zhu et al., 2005), and inwardly rectifying due to reduction in cooperation between subconductances (Wang et al., 2008a; Zhu et al., 2005), and suggested that it may...
contribute directly to pacemaking and to stabilising the resting membrane potential of the cell. Conversely, Parsons and Sanders (2008) found that an HCCC was outwardly rectifying and Ca\(^{2+}\)-independent, and suggested the current may act to increase the excitability of ICC immediately after repolarisation. Wright et al. (2012) recently clarified that maxi Cl\(^{-}\) currents in cultured ICC are inwardly rectifying when recorded in cell-attached patches but outwardly rectifying in the inside-out patch configuration, and confirmed that the maxi Cl\(^{-}\) channel is activated by increases in [Ca\(^{2+}\)].

### Maxi Channels

Parsons et al. (2012) observed a high-conductance channel in murine ICC-MY in situ with similar conductance and rectification properties to the HCCC observed in cultured ICC (Huizinga et al., 2002; Parsons and Sanders, 2008; Zhu et al., 2005; Wright et al., 2012). However, the in situ high-conductance channel had a Na\(^{+}\) to Cl\(^{-}\) permeability ratio (P\(_{\text{Na}}\)/P\(_{\text{Cl}}\)) of 0.76 to 1.64, giving a physiological reversal potential close to 0 mV, so Parsons et al. (2012) renamed the in situ channel a ‘maxi-channel’ and suggested it is either a different protein or regulated differently to the HCCC observed in culture.

### Volume-Activated Chloride Channel

Park et al. (2005) identified a volume-activated Cl\(^{-}\) current in ICC cultured from mouse small intestine. The whole cell current was Ca\(^{2+}\)-independent, outwardly rectifying, and appeared to contribute to the maintenance of RMP and increased excitability of the plateau phase during stretch.

### A Note on Cl\(^{-}\) Equilibrium Potential

Cl\(^{-}\) currents in ICC are likely to reverse during the slow wave, but the exact Cl\(^{-}\) current reversal potential is disputable. The reversal potentials of Cl\(^{-}\) currents recorded from cell-attached patches in mouse small intestine ICC range from −27 mV (Wang et al., 2008a) to −64 mV (D’Antonio et al., 2009). Zhu et al. (2010) measured intracellular Cl\(^{-}\) concentration ([Cl\(^{-}\)]\(_{i}\)) at known values of extracellular Cl\(^{-}\) ([Cl\(^{-}\)]\(_{o}\)) in ICC-MY from mouse small intestine, and found that Cl\(^{-}\) equilibrium potential (E\(_{\text{Cl}}\)) was −47 mV to
−52 mV in situ and −41 mV in culture, supporting the theory that Cl− currents are inward (depolarising) at RMP and outward (repolarising) during the plateau phase. On the other hand, low [Cl−]o reduced the duration of pacemaker potentials in both mouse small intestine and guinea-pig gastric antrum, suggesting that inward Cl− currents contribute to the plateau phase (Kito et al., 2002a; Kito and Suzuki, 2003). It should be noted that both patch-clamp and intracellular recording techniques have the potential to change intracellular ion concentrations and equilibrium potentials, as mentioned in Section 2.3.1.

Interestingly, Zhu et al. (2010) showed that ICC in situ modulate [Cl−]i to maintain ECl close to −50 mV. Similarly, ECl in vascular SMC is approximately 20 mV to 30 mV more positive than RMP due to active accumulation of Cl− ions by ion transporters (Forrest et al., 2010). [Cl−]i in ICC may be controlled by the Na-K-2Cl cotransporter isoform 1 (NKCC1), which typically acts to maintain [Cl−]i above electrochemical equilibrium (Russell, 2000). NKCC1 is expressed in ICC-MY but not ICC-DMP, and appears to be involved in the pacemaker mechanism (Wouters et al., 2006).

Pharmacological Evidence for the Role of Cl− Channels in ICC

Attempts to determine precisely how Cl− channels contribute to pacemaker activity typically involve applying Cl− channel blockers and noting which phases of pacemaker potentials and slow waves are affected (Hirst et al., 2002a; Kito et al., 2002a; Kito and Suzuki, 2003). However, all the Cl− channel blockers used have non-specific effects, as detailed in Section 2.3.4, notably inhibition of NSC currents (Gögelein et al., 1990; Takeda et al., 2008), stimulation of K+ currents (Farrugia et al., 1993; Ottolia and Toro, 1994), causing Ca2+ release from the sarcoplasmic reticulum (SR) (Cruickshank et al., 2003), and potentiation of Ca2+-activated Cl− channels by niflumic acid (Piper et al., 2002). In addition, the effects of Cl− channel blockers on ICC pacemaker activity vary depending on the tissue preparation and drug used.

Nevertheless, Cl− channel blockers do inhibit Cl− currents in ICC. Niflumic acid decreased the amplitude of a Ca2+-activated Cl− current and slowed its deactivation in isolated mouse small intestine ICC (Zhu et al., 2009). The HCCC current was reduced in amplitude by DIDS or 9-AC (Zhu et al., 2005) and blocked completely by DIDS or
SITS (Huizinga et al., 2002) in cultured mouse intestine ICC. On the other hand, the volume-activated Cl\(^-\) current was inhibited by DIDS at +80 mV membrane potential, but not at −80 mV (Park et al., 2005).

The variable effects of Cl\(^-\) channel blockers on ICC activity have been reviewed in detail (Lees-Green et al., 2011b), and will be summarised here. Cl\(^-\) channel blockers and reduced \([\text{Cl}^-]_o\) tended to reduce frequency (Hwang et al., 2009; Kito et al., 2002a; Kito and Suzuki, 2003; Zhu et al., 2009), with the exceptions that frequency changes were not observed in most experiments with guinea-pig gastric antrum (Hirst et al., 2002a) and DIDS did not reduce frequency in mouse small intestine tissue (Hwang et al., 2009; Kito and Suzuki, 2003). Cl\(^-\) channel blockers and low \([\text{Cl}^-]_o\) also tended to reduce slow wave and pacemaker potential amplitude (Hirst et al., 2002a; Hwang et al., 2009; Kito and Suzuki, 2003), with the caveat that reductions were often not seen until high drug concentrations were applied (Hwang et al., 2009). Cl\(^-\) channel blockers and low \([\text{Cl}^-]_o\) tended to reduce the duration of pacemaker potentials (Kito et al., 2002a; Kito and Suzuki, 2003), although niflumic acid sometimes increased duration (Hirst et al., 2002a; Zhu et al., 2009) and may have been acting as an agonist (Piper et al., 2002).

The robustness of niflumic acid-inhibited currents or their role in ICC pacemaker mechanisms appeared to be highly dependent on species, organ, and experimental preparation. Much higher concentrations of niflumic acid were required to inhibit slow wave activity in mouse small intestine tissue (Hwang et al., 2009) than in isolated murine small intestine ICC (Zhu et al., 2009). Furthermore, mouse small intestine slow waves were much less sensitive to niflumic acid than mouse gastric slow waves, and human intestine was much less sensitive than mouse intestine (Hwang et al., 2009).

Cl\(^-\) channels, in particular Ano1, do seem to be important for slow wave production, but Cl\(^-\) channel blockers have too many varying and non-specific effects to aid in elucidating the exact role of Cl\(^-\) currents in ICC.

### 2.6.4 Non-Selective Cation Channels

Koh et al. (2002) observed a Ca\(^{2+}\)-inhibited NSC current in cultured murine ICC that
activated periodically, leading Sanders et al. (2006) to propose that the Ca\textsuperscript{2+}-inhibited NSC channel generated the pacemaker current.

Takeda et al. (2008) identified two different NSC currents in freshly dispersed ICC from mouse gastric antrum. A basally active, Ca\textsuperscript{2+}-inhibited current was found in cells that resembled ICC-MY, while a Ca\textsuperscript{2+}-facilitated current that was responsible for generating noisy spontaneous transient inward currents (STICs) was found in cells that resembled ICC-IM. The Ca\textsuperscript{2+}-inhibited current, putatively observed in ICC-MY, was a large basal inward current that decreased in magnitude when [Ca\textsuperscript{2+}]\textsubscript{i} was increased secondary to depolarisation, and this was shown to be mediated by calmodulin. The current was identified as a NSC current because it had a reversal potential near 0 mV and was inhibited by removing extracellular Na\textsuperscript{+} (Takeda et al., 2008).

The cells believed to be ICC-IM generated basally active STICs, which appeared to summate during depolarisation-induced increases in [Ca\textsuperscript{2+}]\textsubscript{i} to produce the larger Ca\textsuperscript{2+}-facilitated current in a calmodulin-dependent manner. The Ca\textsuperscript{2+}-facilitated current was also identified as a NSC current and was inhibited by Cl\textsuperscript{−} channel blockers (Takeda et al., 2008).

Ca\textsuperscript{2+}-activated NSC currents have also been found in freshly dispersed ICC from murine small intestine. Goto et al. (2004) called this current an autonomous inward current because it was activated upon depolarisation of the cell and then followed a time course that was largely independent of the subsequent voltage steps applied. Goto et al. (2004) speculated that the autonomous inward NSC current was activated by increases in [Ca\textsuperscript{2+}]\textsubscript{i} secondary to membrane depolarisation, but did not provide direct evidence of Ca\textsuperscript{2+}-dependence.

There were similarities between the autonomous inward current (Goto et al., 2004) and the Ca\textsuperscript{2+}-facilitated current (Takeda et al., 2008). Both were activated by depolarisation, which was believed to be due to depolarisation-induced increases in [Ca\textsuperscript{2+}]\textsubscript{i}. In addition, both the autonomous inward current and the Ca\textsuperscript{2+}-facilitated current had linear conductance and demonstrated large tail currents on repolarisation. Therefore, it is possible that these two currents were generated by the same type of NSC channel.

The channels responsible for NSC currents in ICC have not been conclusively identified.
A candidate for NSC channels is the family of TRP homologues. At least four types of TRPC and two types of TRPM are expressed in ICC from mouse small intestine and stomach (Chen et al., 2007; Epperson et al., 2000; Liu et al., 2005a). TRPC4 was proposed to generate the pacemaker current (Torihashi et al., 2002; Walker et al., 2002), but TRPC4−/− mice were subsequently shown to display normal slow waves (Kim et al., 2006b). TRPM7 was also proposed as a pacemaker channel (Kim et al., 2005, 2009a), although mechanisms of activation of TRPM7 remain unknown (Wu et al., 2010).

In light of the recent discoveries about the role of TRP channels as non-selective SOC channels (Ng et al., 2009; Yuan et al., 2012), some of the NSC currents observed in ICC may also be involved in SOCE.

### 2.6.5 Potassium Channels

A wide range of K+ channels are expressed in excitable cells, and many types have been found in ICC, including Ca2+-activated K+ channels, inward rectifier K+ channels, and a delayed rectifier K+ channel. K+ channels generate outward current at physiological potentials, and can contribute to slow wave repolarisation, balancing inward current during the plateau phase, or maintaining the resting membrane potential to control ICC excitability.

**Calcium-Activated Potassium Channels**

Small (SK), intermediate (IK) and large (BK) conductance Ca2+-activated K+ channels have been found in ICC. BK channels proteins (Cho and Daniel, 2005; Zhu and Huizinga, 2008) and RNA (Chen et al., 2007) have been identified in ICC from murine small intestine, and a large conductance (218 pS) Ca2+-activated K+ current was recorded from canine colonic ICC (Langton et al., 1989). Zhu and Huizinga (2008) proposed that the primary role of BK channels may be to regulate ICC excitability, particularly during the plateau phase, in response to neurotransmitters and other chemical stimuli, because nitrergic innervation appears to inhibit ICC by activating BK channels.

An IK current with 38 pS single channel conductance was identified in murine jejunal...
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ICC *in situ* and in culture (Zhu et al., 2007). This study suggested that IK may regulate ICC excitability, because blocking the current depolarised RMP and increased slow wave duration. The current was enhanced by nitric oxide, so it may also play a role in mediating nitrergic innervation. An SK channel, SK3, is expressed in ICC-MY and ICC-IM in the stomach, small intestine and colon of rats (Fujita et al., 2001).

**K*V*1.1 Delayed Rectifier Potassium Channel**

*K*V*1.1 channels generate a delayed rectifier K⁺ current. K*V*1.1 is expressed in ICC from dog, guinea-pig, and mouse, and a K*V*1.1 blocker, dendrotoxin-K, blocked part of a delayed rectifier outward current in ICC cultured from mouse gastric fundus (Hatton et al., 2001). K*V*1.1 channels may contribute to repolarisation and ICC excitability (Huizinga et al., 2004).

**Inward Rectifier Potassium Channels**

Ether-à-go-go related gene (ERG) K⁺ channels are found in ICC. ERG1 was abundantly expressed in murine small intestine ICC-MY (Chen et al., 2007), while ERG3 has been found in the same cell type in culture (White et al., 2008). ERG current in cultured murine small intestine ICC was found to activate on depolarisation then rapidly inactivate to a smaller sustained current, and to reactivate upon repolarisation (McKay et al., 2006). ERG currents contribute to the slow wave plateau, because the ERG window current is maximal around −30 mV (McKay et al., 2006), and blocking ERG with E4031 leads to increased slow wave duration (McKay et al., 2006; White et al., 2008; Zhu et al., 2003). ERG currents act as inward rectifiers on hyperpolarisation from potentials positive to RMP (Zhu et al., 2003). ERG current also regulates RMP because E4031 depolarises RMP (White et al., 2008; Zhu et al., 2003).

Another inward-rectifier K⁺ current thought to be present in ICC is an ATP-sensitive K⁺ current. This current is activated by phentolamine (Ahn et al., 2010), pinacidil (Choi et al., 2006), and hydrogen peroxide (Choi et al., 2010). These drugs have been shown to inhibit pacemaker currents, increase outward currents, cause hyperpolarisation, and decrease slow wave frequency in ICC from mice and guinea-pigs (Ahn et al., 2010; Choi
et al., 2006, 2010; Kito et al., 2002b, 2005). These actions were blocked by glibenclamide, which inhibits ATP-sensitive K\(^{+}\) currents (Ahn et al., 2010; Choi et al., 2010), indicating that ATP-sensitive K\(^{+}\) channels are present in the cell membrane. However, the frequency and amplitude of unitary potentials was increased by pinacidil (Kito et al., 2002b), and glibenclamide blocked internal Ca\(^{2+}\) cycling (Fukuta et al., 2002), suggesting a role for ATP-sensitive K\(^{+}\) channels in the mitochondrial membrane.

### Transient Outward Potassium Channel

Parsons and Huizinga (2010) identified a transient outward K\(^{+}\) current that activated immediately on depolarisation from RMP in ICC cultured from murine small intestine and proposed that it may play a role in regulating the slow wave upstroke phase.

### Background Potassium Channel

KCNK3, which encodes the K\(_{2P}\)-3.1 two-pore domain potassium channel, also known as TASK-1, is expressed in ICC-MY. K\(_{2P}\)-3.1 carries a background or leak K\(^{+}\) current, and probably contributes to maintaining RMP and regulating excitability (Chen et al., 2007; Goldstein et al., 2005). KCNK3 is more abundantly expressed in ICC-MY than in ICC-DMP (Chen et al., 2007), and in W/W\(^{V}\) mice without ICC-MY the resting membrane potential of the smooth muscle tissue is around 9 mV depolarised compared with wild-type mice (Ward et al., 1994), suggesting that KCNK3 sets RMP at a lower level in ICC-MY than the intrinsic RMP of ICC-DMP and SMC.

### 2.7 Unitary Potentials

In addition to generating slow waves, ICC generate spontaneous transient depolarisations up to 10 mV in amplitude (Edwards et al., 1999; Hirst and Edwards, 2001; Kito et al., 2002b). Suzuki and Hirst (1999) first observed that membrane potential recordings from circular muscle bundles from the guinea-pig gastric antrum were characterised by a constant discharge of noise. Edwards et al. (1999) found that applying a low concentration of the Ca\(^{2+}\) chelator BAPTA-AM (20 \(\mu\)M) reduced the frequency of occurrence of spontaneous
depolarisations, enabling them to resolve discrete events, which they called unitary potentials. Unitary potentials have also been observed in ICC-MY in the intervals between pacemaker potentials recorded from the guinea-pig gastric antrum (Kito et al., 2002b) and mouse small intestine (Kito and Suzuki, 2003). Although the term unitary potentials is widely used in the ICC literature (e.g. Jin et al., 2009; Kito et al., 2005), analogous events in non-GI ICC and SMC are typically referred to as spontaneous transient depolarisations generated by spontaneous transient inward currents (STICs) (Sergeant et al., 2001; van Helden, 1991; Wang et al., 1992). This terminology is more recently gaining traction in the GI ICC field (Kito, 2011; Zhu et al., 2011).

Unitary potentials exhibit both $\text{Ca}^{2+}$ and voltage dependence. $\text{Ca}^{2+}$ dependence was evidenced by the decreased frequency of unitary potentials caused by BAPTA-AM (Edwards et al., 1999; Kito et al., 2002b), and the reduced amplitude of unitary potentials in the presence of caffeine (Edwards et al., 1999). In ICC-MY from mouse small intestine the presence of either 50 $\mu$M BAPTA-AM or nominally $\text{Ca}^{2+}$-free solution enabled unitary potentials to be observed more frequently, but it was unclear whether this was due to inhibition of pacemaker potentials or facilitation of unitary potentials (Kito and Suzuki, 2003).

The voltage dependence of unitary potentials was evidenced by the response of unitary potentials to changes in membrane potential. Edwards et al. (1999) noted that unitary potentials were evoked during depolarisation and following hyperpolarisation of circular muscle bundles. Kito et al. (2002b) and Kito and Suzuki (2003) showed that depolarising membrane potential using high $[\text{K}^+]_{o}$ solution caused unitary potentials to appear more frequently in ICC-MY from guinea-pig gastric antrum and mouse small intestine, while hyperpolarising guinea-pig gastric antrum ICC-MY by opening ATP-sensitive $\text{K}^+$ channels also increased the frequency and amplitude of unitary potentials.

Using spectral density curves, Edwards et al. (1999) showed that the frequency profile of membrane potential recordings was similar during baseline and during the falling phase of both regenerative potentials and spontaneously occurring slow waves, suggesting that each type of recording consisted of the same underlying events. Their observations led Edwards et al. (1999) to propose that regenerative potentials were produced by the
summation of many unitary potentials, and that unitary potentials reflect the coordinated activity of several sets of Ca\(^{2+}\)-activated ion channels within a single cell or multiple neighbouring cells.

The spectral density curves of the plateau component of pacemaker potentials from guinea-pig gastric antrum ICC-MY (Hirst and Edwards, 2001) had the same temporal characteristics as those from circular muscle bundles (Edwards et al., 1999), indicating that the pacemaker potential plateau was also largely composed of unitary potentials. In addition, Hirst and Edwards (2001) observed that the magnitude of membrane noise or spontaneous transient depolarisations was smallest immediately following a pacemaker potential and largest immediately before a pacemaker potential, with a roughly linear increase in magnitude over the course of the diastolic interval between pacemaker potentials. Hirst and Edwards (2001) thus proposed that a discharge of unitary potentials initiates the upstroke component of pacemaker potentials, which triggers ongoing activation of unitary potentials to form the plateau component. Conversely, in mouse small intestine the primary upstroke component and the plateau component of pacemaker potentials could be abolished independently by the application of Ni\(^{2+}\) and DIDS, respectively, so Kito and Suzuki (2003) reasoned that pacemaker potentials may be initiated by a different mechanism, or that the primary and plateau components may be generated by different populations of unitary potentials.

The currents underlying unitary potentials are STICs, which in vascular SMC are carried by Ca\(^{2+}\)-activated Cl\(^{-}\) channels (Wang et al., 1992). In ICC, STICs can be generated by Ca\(^{2+}\)-inhibited NSC channels (Jin et al., 2009), Ca\(^{2+}\)-activated NSC channels (Takeda et al., 2008), and Ca\(^{2+}\)-activated Cl\(^{-}\) channels (Zhu et al., 2011).

In summary, unitary potentials triggered by stochastic Ca\(^{2+}\) release from intracellular stores are the fundamental pacemaker events that initiate pacemaker potentials (Edwards et al., 1999; Hirst and Edwards, 2001). Unitary potentials are also thought to form the plateau phase of pacemaker potentials and regenerative potentials, due to a coordinated increase in [Ca\(^{2+}\)].
Pacemaker Units

ICC contain abundant mitochondria, and sections of ER, mitochondria, and cell membrane in close apposition—on the order of 10 nm apart—are proposed to create pockets of cytoplasm partially isolated from the rest of the cell in which localised $[\text{Ca}^{2+}]_i$ dynamics can occur (Koh et al., 2002; Sanders et al., 2006; Ward et al., 2000). Sanders and colleagues called these structures pacemaker units (PMUs or PUs), and suggested that they are the elementary structural origin of unitary potentials, whereby the ion channels that generate unitary potentials are localised to PMUs and are activated by $[\text{Ca}^{2+}]_i$ changes due to $\text{Ca}^{2+}$ cycling between the mitochondria and ER (Sanders et al., 2006).

2.8 Voltage-Dependent Entrainment

Unitary potentials are generated by $\text{Ca}^{2+}$-dependent mechanisms, so a voltage-dependent mechanism may be required to entrain multiple PMUs to fire in a coordinated manner to generate the slow wave plateau phase. IP$_3$Rs can be opened by $\text{Ca}^{2+}$, so Koh et al. (2003) proposed that voltage-dependent $\text{Ca}^{2+}$ influx was responsible for entrainment. A candidate channel was the voltage-dependent, DHP-resistant $\text{Ca}^{2+}$ channel identified by Kim et al. (2002). This channel was inhibited by Ni$^{2+}$ and mibefradil, which also inhibited the upstroke rate and propagation of slow waves, and is now believed to be a T-type $\text{Ca}^{2+}$ channel (Lee et al., 2007b).

The entrainment theory developed by Sanders et al. (2006) states that depolarisation from several concurrent unitary potentials activates nearby T-type $\text{Ca}^{2+}$ channels. The resultant influx of $\text{Ca}^{2+}$ current is responsible for the upstroke depolarisation, and causes an increase in $[\text{Ca}^{2+}]_i$ in PMUs that have not yet fired, initiating new unitary potentials by stimulating IP$_3$R-mediated $\text{Ca}^{2+}$ release (Lee et al., 2007b; Sanders et al., 2006).

The same voltage-mediated entrainment mechanism is believed to be responsible for propagating slow waves through ICC networks, because slow waves propagate at least an order of magnitude faster than $\text{Ca}^{2+}$ diffuses (Ward et al., 2003). It has been shown in canine gastric and colonic muscles that slow wave propagation requires $\text{Ca}^{2+}$ entry and reactivation of the internal IP$_3$- and mitochondria-related pacemaker mechanism.
Thus, T-type Ca\(^{2+}\) channels may be responsible for entraining PMUs to generate the slow wave plateau phase and to propagate slow waves through ICC networks.

In ICC-IM, T-type Ca\(^{2+}\) channels appear to be absent, and Hirst et al. (2002a) proposed an alternative mechanism of entrainment in which voltage-dependent oscillations in IP\(_3\) concentration are responsible for entraining pacemaker units. In guinea-pig gastric antrum circular muscle, regenerative potentials occur with a delay of one or more seconds following a change in membrane potential, suggesting that a slow process like voltage-dependent activation of phospholipase C is responsible for synchronising Ca\(^{2+}\) transients and unitary potentials in response to depolarisation (Edwards et al., 1999). Voltage-dependent modulation of IP\(_3\) production occurs in vascular (Ganitkevich and Isenberg, 1993; Itoh et al., 1992) and jejunal SMC (Best and Bolton, 1986). Slow wave entrainment by voltage-dependent IP\(_3\) production has been successfully implemented in a model of ICC-MY (Du et al., 2010c; Imtiaz et al., 2002). However, voltage-dependent changes in [Ca\(^{2+}\)]\(_i\) may also mediate IP\(_3\) concentration and IP\(_3\)R open probability (De Young and Keizer, 1992), and at present there is no experimental evidence in ICC that IP\(_3\) production or degradation is directly mediated by membrane potential.

### 2.9 Pacemaker Hypotheses

#### 2.9.1 The NSCC Pacemaking Hypothesis

The first attempt to assemble the knowledge about Ca\(^{2+}\) handling and ion channels in ICC into a comprehensive theory regarding ICC pacemaker mechanisms was the non-selective cation channel (NSCC) Hypothesis proposed by Sanders et al. (2006). The NSCC Hypothesis (also referred to as the Sanders Hypothesis) posited that Ca\(^{2+}\) release from IP\(_3\)R-operated stores in a PMU initiates the pacemaker cycle by gating open Ca\(^{2+}\) uptake transporters in mitochondria. The mitochondrial Ca\(^{2+}\) uptake is sufficient to decrease [Ca\(^{2+}\)]\(_i\) within the cytosolic subspace of the PMU, thus activating Ca\(^{2+}\)-inhibited NSC channels. The resultant inward pacemaker current would generate unitary potentials (Koh
et al., 2002). In response to the depolarisation from one or several unitary potentials, voltage-dependent Ca$^{2+}$ influx via T-type Ca$^{2+}$ channels located within PMUs was proposed to coordinating unitary potential firing to generate the plateau phase. T-type Ca$^{2+}$ current was also proposed to entrain pacemaker potentials in ICC networks (Sanders et al., 2006). Sanders et al. (2006) suggested that intracellular Ca$^{2+}$ stores were maintained by a mNCX extruding mitochondrial Ca$^{2+}$ to the cytosol, and SERCA pumps taking cytosolic Ca$^{2+}$ back up into the ER.

2.9.2 Recent Developments Challenging the NSCC Hypothesis

Critical challenges to the NSCC Hypothesis have come from both experimental and modelling studies. When the NSCC Hypothesis was developed, the Ca$^{2+}$-inactivated NSC channel seemed to generate a viable pacemaking current, based on experiments using cultured ICC (Koh et al., 1998, 2002; Torihashi et al., 2002). However, phenotypic changes in ICC in culture may include changes in the expression of pacemaker conductances (Sanders et al., 2006; Zhu et al., 2009), and an oscillatory Ca$^{2+}$-inactivated NSC current has not been observed in ICC in situ.

Further research has cast doubt on crucial aspects of the NSCC Hypothesis, including the role of the Ca$^{2+}$-inhibited NSCC (Zhu et al., 2009; Hwang et al., 2009; Means and Sneyd, 2010), and mitochondria (Lowie et al., 2011).

A detailed modelling study of Ca$^{2+}$ transients in PMUs by Means and Sneyd (2010) demonstrated that cyclical generation of unitary potentials by the Ca$^{2+}$-inactivated NSC channel described by Sanders et al. (2006) is not feasible. Firstly, [Ca$^{2+}$]$_i$ in the pacemaker unit could only be reduced below baseline levels using non-physiological Ca$^{2+}$ transporters or when IP$_3$Rs were inactive during ER store depletion. Secondly, half-maximal inactivation of the NSC current occurs when [Ca$^{2+}$]$_i$ is around 375 nM (Faville et al., 2008), which suggests resting [Ca$^{2+}$]$_i$ in the PMU should be higher than this. Means and Sneyd (2010) showed that maintaining [Ca$^{2+}$]$_i$ in the PMU at 400 nM caused [Ca$^{2+}$]$_i$ in the rest of the cell to rise to toxic levels. Therefore, it appears that the Ca$^{2+}$-inhibited NSC channel does not act to generate a pacemaker current, and is more likely to be basally active.
Furthermore, the identification of the Ano1 Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} channel in ICC \textit{in situ} (Gomez-Pinilla et al., 2009) and the discovery that Ano1 is essential for slow wave production (Hwang et al., 2009) provided strong evidence for an alternative to the Ca\textsuperscript{2+}-inhibited NSCC as a pacemaker channel, resulting in calls for a renewed consideration of the ICC pacemaker mechanism (Hwang et al., 2009; Zhu et al., 2009).

### 2.9.3 Calcium-Activated Channels: An Alternative to the NSCC Hypothesis

Prior to the discovery of Ano1 in ICC, Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} currents were also commonly proposed to act as pacemaker currents (Hirst et al., 2002a; Huizinga et al., 2002; Liu et al., 2005a; Tokutomi et al., 1995). Huizinga et al. (2002) recorded rhythmic high-conductance Cl\textsuperscript{−} currents in ICC, and Cl\textsuperscript{−} channel blockers were shown to inhibit slow wave activity (Hirst et al., 2002a; Kito et al., 2002a). However, Cl\textsuperscript{−} channel blockers also inhibited the NSC current (Koh et al., 2002), and until recently the Ca\textsuperscript{2+}-dependence of the Cl\textsuperscript{−} currents observed in ICC was disputed (Parsons and Sanders, 2008). It is only in the last several years that a Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} current was conclusively shown to be a plausible pacemaker current, with the discovery of Ano1 in ICC \textit{in situ} (Gomez-Pinilla et al., 2009) and confirmation that the HCCC or maxi Cl\textsuperscript{−} channel was activated by intracellular Ca\textsuperscript{2+} (Wright et al., 2012).

Ca\textsuperscript{2+}-facilitated NSC currents have also been proposed as primary pacemaker currents in ICC, based on recordings of a current in freshly prepared ICC that followed an autonomous time-course and reversed around 3 mV (Goto et al., 2004).

Pharmacological agents are routinely used to determine the role of different ion currents, but the blockers used to inhibit Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} channels in ICC also inhibit NSCC (Gögelein et al., 1990; Takeda et al., 2008), so it is difficult to conclusively prove that either Cl\textsuperscript{−} channels or NSC channels are exclusively responsible for pacemaker currents. Additionally, Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} channels are likely to generate outward repolarising current during the plateau phase of pacemaker potentials (Zhu et al., 2010), whereas NSC channels generate an inward, depolarising current that would maintain the membrane
potential of ICC at a depolarised level during the peak plateau phase of the slow wave. In light of recent developments we proposed an alternative pacemaker hypothesis in which both Ano1 channels and Ca\textsuperscript{2+}-activated NSC channels may initiate pacemaker potentials in response to localised IP\textsubscript{3}-mediated Ca\textsuperscript{2+} release (Lees-Green et al., 2011b). Our pacemaker mechanism was in many other respects similar to the NSCC Hypothesis, with Ca\textsuperscript{2+} influx through T-type Ca\textsuperscript{2+} channels coordinating unitary potentials to generate the plateau phase.

In summary, candidate pacemaker currents include Ano1 Ca\textsuperscript{2+}-activated Cl\textsuperscript{-} channels (Hwang et al., 2009; Zhu et al., 2009), Ca\textsuperscript{2+}-activated maxi Cl\textsuperscript{-} currents (Wright et al., 2012), Ca\textsuperscript{2+}-activated NSC channels (Goto et al., 2004), and TRPM7 NSC channels (Kim et al., 2011). The exact mechanism of interaction between these ion channels and intracellular Ca\textsuperscript{2+} oscillations remains open to speculation, but what we do know is summarised in Section 2.9.4.

### 2.9.4 Revising the ICC Pacemaker Mechanisms

For reasons detailed in Sections 2.9.1–2.9.3, serious reservations now exist regarding the previously dominant NSCC Hypothesis for ICC pacemaking, although the importance of intracellular Ca\textsuperscript{2+} handling in the pacemaker process remains clear. There are four major contentions for resolution: i) What are the specific membrane ion channels involved in ICC pacemaking?; ii) Are pacemaker channels activated by the rising or falling phase of the internal Ca\textsuperscript{2+} oscillations?; iii) How do the various Ca\textsuperscript{2+} sources contribute to the Ca\textsuperscript{2+} oscillations? and iv) How do ion channels other than the pacemaker channel contribute to slow wave production and propagation?

Based on the evidence outlined in Sections 2.4–2.8, slow waves are generated by the process described below. The Ca\textsuperscript{2+} handling mechanisms underlying the pacemaker cycle are summarised in Figure 2.6, and the ion channels thought to contribute to each phase of the slow wave are summarised in Figure 2.7.

The pacemaker cycle is initiated by stochastic Ca\textsuperscript{2+} release from IP\textsubscript{3}Rs on the ER (Hirst and Edwards, 2001; Suzuki et al., 2000; Ward et al., 2000). IP\textsubscript{3}R-mediated Ca\textsuperscript{2+}
Figure 2.6: The calcium channels and transporters that may underlie the pacemaker cycle in ICC-MY. The contributions of components marked with a ‘?’ remain unresolved. SERCA pumps and IP3Rs control Ca^{2+} cycling between the ER and the cytosol. Mitochondrial Ca^{2+} uptake via uniporters (uni) may prolong IP3R-mediated Ca^{2+} release, with Ca^{2+} being returned to the cytosol by mNCX. The identity of the pacemaker channel needs to be confirmed, and the phase of the Ca^{2+} cycle that activates the pacemaker channel (e.g. IP3R Ca^{2+} release or SOCE) is also subject to debate. Ca^{2+} influx through SOC channels may also regulate the pace of Ca^{2+} cycling. A voltage-dependent Ca^{2+} influx through T-type Ca^{2+} channels contributes to the upstroke phase and can entrain the pacemaker cycle. PMCA pumps maintain Ca^{2+} homeostasis in the cytosol.

release may cause a localised increase in [Ca^{2+}], that directly activates a pacemaker current to generate a unitary potential (Lees-Green et al., 2011b; van Helden and Imtiaz, 2003). Alternatively, the corresponding decrease in [Ca^{2+}]_{ER} may cause SOC channels to open, enabling the pacemaker current to be activated by a localised Ca^{2+} influx through SOC channels (Lees-Green et al., 2014). While SOCE clearly occurs in ICC (Daniel et al., 2009; Liu et al., 2005a; Torihashi et al., 2002), the non-specificity of 2-APB (Bootman et al., 2002) means that SOCE has not been conclusively linked to a particular phase of the pacemaker cycle. SOCE may act to initiate pacemaker potentials, but it may also act to replenish the ER following a pacemaker potential and thus help to set the pace of Ca^{2+} cycling (Daniel et al., 2009; Means and Sneyd, 2010). SERCA pumps in the ER and
plasma membrane calcium ATPase (PMCA) pumps in the plasma membrane are likely to complete the Ca\(^{2+}\) cycle by removing Ca\(^{2+}\) from the cytosol (Means and Sneyd, 2010). Mitochondrial Ca\(^{2+}\) handling may play a role in ICC pacemaking, but the role of mitochondria is no longer as evident as it was in the NSCC Hypothesis. In cultured ICC, inhibiting mNCX was found to inhibit pacemaker currents and slow waves (Kim et al., 2006a), but a more recent study on ICC in situ found that mitochondrial Ca\(^{2+}\) release via mNCX is not important for Ca\(^{2+}\) cycling (Lowie et al., 2011).

In cardiac myocytes, the main role for mitochondria is ATP synthesis to power contraction and SERCA pumps (Lukyanenko et al., 2009), and mitochondrial Ca\(^{2+}\) uptake and extrusion mechanisms were traditionally considered too slow to contribute to pacemaker activity (Griffiths et al., 2010). However, interactions between Ca\(^{2+}\) and mitochondria are important for regulating ATP supply and modulating Ca\(^{2+}\) signalling and homeostasis, and more recent evidence suggests that mitochondria proximal to Ca\(^{2+}\) stores can also contribute directly to [Ca\(^{2+}\)]\(_i\) transients during excitation-contraction coupling (Griffiths et al., 2010). Mitochondria may similarly have multiple important roles in ICC. Mitochondria can indirectly contribute to Ca\(^{2+}\) cycling by generating ATP to power the SERCA pump, and by contributing to Ca\(^{2+}\) homeostasis through slow mitochondrial Ca\(^{2+}\) uptake and release processes. At the same time, faster mitochondrial Ca\(^{2+}\) cycling may have a direct effect on [Ca\(^{2+}\)]\(_i\) transients during the pacemaker cycle, as previously suggested. In colonic SMC, mitochondrial Ca\(^{2+}\) uptake helps to reduce the inhibitory effect of high [Ca\(^{2+}\)]\(_i\) (> 500 nM) on IP\(_3\)Rs (Chalmers and McCarron, 2009; Olson et al., 2010), and it may have a similar effect in ICC, prolonging ER Ca\(^{2+}\) release and the duration of the pacemaker current, but further definitive research on the role of mitochondria in ICC Ca\(^{2+}\) cycling is still required.

Both Ca\(^{2+}\)-activated Cl\(^-\) currents and NSC currents have been implicated as the pacemaker current in ICC (Goto et al., 2004; Wright et al., 2012; Zhu et al., 2009) and shown to generate STICs (Jin et al., 2009; Takeda et al., 2008; Zhu et al., 2011). Wang et al. (2008a) observed that the reversal potential of spontaneous inward currents in mouse and rat ICC pointed to a role for both Cl\(^-\) channels and NSC channels in pacemaking. Unfortunately, pharmacological studies cannot readily differentiate between Cl\(^-\) and NSC currents (Gögelein et al., 1990; White and Aylwin, 1990). However, Ano1 channels are
likely to contribute as a pacemaker channel at RMP because Cl\(^-\) currents are thought to reverse above \(-41\) mV to \(-52\) mV (Zhu et al., 2010), and a Ca\(^{2+}\)-activated Cl\(^-\) current in murine intestinal ICC was found to activate at \(-72\) mV (Zhu et al., 2009). In addition, the absence of slow waves in smooth muscle tissue from Ano1 knockout mice suggests that NSC channels alone cannot initiate pacemaker potentials (Hwang et al., 2009).

The depolarisation from one or several unitary potentials activates T-type Ca\(^{2+}\) channels (Lee et al., 2007b), resulting in a large influx of Ca\(^{2+}\) and a rapid depolarisation forming the slow wave upstroke phase. In human ICC, Na\(_V\)1.5 channels are also likely to contribute to the upstroke depolarisation (Strege et al., 2003b). The rise rate of slow waves is reduced by mibebradil (Kito et al., 2005), which inhibits both T-type Ca\(^{2+}\) and Na\(_V\)1.5 currents (Strege et al., 2005). T-type Ca\(^{2+}\) channels (Perez-Reyes, 2003) and Na\(_V\)1.5 channels (Strege et al., 2003b) both activate around \(-60\) mV to \(-50\) mV, peak around \(-30\) mV to \(-20\) mV, and are fast-inactivating, making them prime candidates for producing the upstroke phase.

Voltage-dependent Ca\(^{2+}\) influx through T-type Ca\(^{2+}\) channels enables the coordinated propagation of slow waves by entraining the Ca\(^{2+}\) cycle in connected ICC (Park et al., 2006; Ward et al., 2004). Ano1 channels are more sensitive to Ca\(^{2+}\) at more positive membrane potentials (Xiao et al., 2011; Yang et al., 2008), so voltage-dependent entrainment may also be due in part to an increase in Ano1 channel activation (Lees-Green et al., 2011b).

Ca\(^{2+}\) influx through T-type Ca\(^{2+}\) channels during the upstroke phase has also been hypothesised to entrain previously quiescent PMUs within a single ICC by opening IP\(_3\)Rs to trigger more unitary potentials (Sanders et al., 2006). Regenerative potentials in ICC-IM are produced by the summation of unitary potentials in an apparently Ca\(^{2+}\)- and voltage-dependent manner (Edwards et al., 1999), and the generation of the pacemaker potential plateau phase may be due to a similar mechanism (Hirst and Edwards, 2001). This is supported by evidence that inhibiting SERCA pumps with CPA or IP\(_3\)Rs with caffeine shortened the duration of pacemaker potentials, suggesting that intracellular Ca\(^{2+}\) handling is essential for generating the plateau phase (Hirst and Edwards, 2001; Kito et al., 2002a). Pharmacological experiments also showed that DIDS or CPA inhibited the plateau component while leaving the initial upstroke intact, whereas Ni\(^{2+}\) or high [K\(^+\)].
solution inhibited the upstroke phase, suggesting that the upstroke and plateau components are generated by independent mechanisms (Kito et al., 2002a; Kito and Suzuki, 2003). Kito and Suzuki (2003) proposed that the initial and plateau components of pacemaker potentials may be produced by two different populations of PMUs. Ano1 channels may initiate pacemaker potentials but Cl\(^{-}\) channels generate outward (repolarising) currents positive to \(-50\) mV (Zhu et al., 2010) so depolarising unitary potentials during the plateau phase may be generated by NSC channels (Lees-Green et al., 2011b).

Other studies suggested that the plateau phase is generated by inward (depolarising) Cl\(^{-}\) currents (Kito et al., 2002a; Kito and Suzuki, 2003; Zhu et al., 2005), although it now appears unlikely that Cl\(^{-}\) currents can be depolarising at the plateau potential (Zhu et al., 2010). Nevertheless, we cannot be certain that the pacemaker potential plateau is generated by unitary potential summation. The plateau phase is likely to be maintained by a balance of inward NSC currents and outward Cl\(^{-}\) and K\(^{+}\) currents, and the identities of the channels active during the plateau have yet to be established.

Slow wave repolarisation occurs by inactivation of inward currents and activation of outward K\(^{+}\) currents toward the end of the plateau phase. Delayed rectifier K\(_{V1.1}\) channels and ERG K\(^{+}\) channels in particular probably act during the repolarisation phase (Beyder and Farrugia, 2012; Huizinga et al., 2004). Slowly deactivating Ano1 channels may also contribute to repolarisation.

The RMP in ICC is typically close to the K\(^{+}\) equilibrium potential (E\(_{K}\)) because of background K\(^{+}\) currents (Chen et al., 2007; Goldstein et al., 2005) tempered by a smaller Na\(^{+}\) leak current (Kim et al., 2012). Inward rectifier ERG K\(^{+}\) currents (Zhu et al., 2003) and high-conductance Cl\(^{-}\) currents (Zhu et al., 2005) may also help to maintain RMP.

Many components of the pacemaker cycle described here are consistent across different species, organs, and types of ICC. However, tissue-specific Na\(_{V1.5}\) expression in the GI tract (Strege et al., 2007) shows that the common pacemaker mechanism can differ across experimental models.

Disparities in the ion channels expressed in murine small intestine ICC-MY and ICC-DMP may explain why only ICC-MY can generate spontaneous pacemaker potentials without neural input, particularly if these differences are replicated in guinea-pig gastric
2.9. Pacemaker Hypotheses

Figure 2.7: A theoretical slow wave trace from human small intestine, indicating the ion currents thought to contribute to the initiation, upstroke phase, plateau phase, repolarisation, and resting potential of slow waves. A downward arrow indicates an inward current, while an upward arrow indicates an outward current. $I_{\text{ClCa}}$: $\text{Ca}^{2+}$-activated $\text{Cl}^{-}$ current; $I_{\text{SOC}}$: store-operated $\text{Ca}^{2+}$ current; $I_{\text{Nav}}$: $\text{NaV}_{1.5}$ current; $I_{\text{CaT}}$: T-type $\text{Ca}^{2+}$ current; $I_{\text{NSCC}}$: $\text{Ca}^{2+}$-activated NSC current; $I_{\text{BK}}$: $\text{Ca}^{2+}$-activated $\text{K}^{+}$ current; $I_{\text{KERG}}$: ERG $\text{K}^{+}$ current; $I_{\text{Kv1.1}}$: delayed-rectifier $\text{K}^{+}$ current; $I_{\text{Kb}}$: background $\text{K}^{+}$ current; $I_{\text{Nab}}$: background $\text{Na}^{+}$ current; $I_{\text{ClHC}}$: high-conductance $\text{Cl}^{-}$ current.

antrum ICC-MY and ICC-IM. $\text{CaV}_{3.2}$ T-type channels are more abundantly expressed in ICC-MY than ICC-DMP (Chen et al., 2007), consistent with a decreased ability of ICC-IM to coordinate unitary potentials to produce pacemaker potentials (Edwards et al., 1999; Kito et al., 2002b). Ano1 is highly expressed in all classes of ICC (Gomez-Pinilla et al., 2009), but $\text{Cl}^{-}$ transporters are expressed more highly in ICC-MY than in ICC-DMP (Chen et al., 2007; Wouters et al., 2006). Without $\text{Cl}^{-}$ transporters, particularly NKCC1, the $E_{\text{Cl}}$ in ICC-DMP is likely to be lower than that in ICC-MY, thus impairing the ability of Ano1 currents to depolarise the cell.

The information compiled here regarding ICC pacemaker mechanisms was utilised to develop a novel model of ICC pacemaker activity, which is presented in Chapter 6.
Chapter 3

Previous Models of ICC Activity


As experimental data on ICC electrophysiology continues to accumulate, it is imperative to coherently integrate this knowledge in order to form, test and extend hypotheses on whole cell function. Biophysically-based models provide an ideal method to achieve this integration.

Mathematical cell models can generally be classified as either phenomenological or biophysical (Cheng et al., 2010). Prior to the discovery that ICC act as pacemaker cells, phenomenological models were used for many years to study the electrical activity of the GI tract. These typically modelled slow waves using relaxation oscillator models in which a system of ordinary differential equations (ODEs) produced oscillatory patterns that match the frequency and duration of slow waves, but the equations could not be directly related to an anatomical or physiological basis for slow waves (Nelsen and Becker, 1968; Publicover and Sanders, 1989; Sarna et al., 1971).

In silico studies using biophysical cell models can simulate experimental conditions by adjusting appropriate parameter values. Cell models can be used to investigate how subcellular phenomena affect slow wave activity, and can be incorporated into multiscale models to study slow wave behaviour at the tissue, organ, and body scales. Potential applications for such simulations include evaluating physical variables not easily assessed experimentally, quantitatively relating structure with function, and potentially reducing experimental animal usage (e.g., Du et al., 2010a,c; Poh et al., 2012).
3. PREVIOUS MODELS OF ICC ACTIVITY

3.1 Phenomenological Cell Models

Phenomenological models had a significant impact on early theories of slow wave propagation and entrainment (Publicover and Sanders, 1989). Nelsen and Becker (1968) published one of the earliest simulations of slow wave activity of the small intestine, consisting of a series of coupled relaxation oscillators. This model applied a generalised version of the van der Pol (1926) oscillator, transformed into a system of two first order differential equations with a stimulus term added to one equation. The morphology and frequency of the simulated slow waves could be controlled by adjusting the parameter values to match experimental data. Sarna et al. (1971) expanded on the concept of coupled oscillators adopted by Nelsen and Becker (1968), and demonstrated entrainment of slow waves to an ‘intact frequency’ in a linear network of coupled oscillators by incorporating forward, backward and phase-shifted backward couplings. However, the applications of relaxation oscillator slow wave models were ultimately limited by the absence intracellular details, particularly ion channels. Consequently, Publicover and Sanders (1989) discussed the limitations of the relaxation oscillator based models, including their inability to represent the effects of pharmacological agents or electrical stimuli on slow wave activity, and the mismatches between the morphologies of simulated slow wave activity and intracellular slow wave recordings. Phenomenological models have now been superseded by more sophisticated modelling methods providing a more physiologically realistic representation of slow waves (Cheng et al., 2010).

3.2 Biophysical Cell Models

Biophysical cell models are usually based on the mathematical approach developed by Hodgkin and Huxley (1952), allowing individual ion currents to be quantitatively evaluated under effects of parameters with meaningful physical quantities, such as temperature, ion concentration, and voltage. Hodgkin-Huxley type models represent the plasma membrane lipid bilayer as a capacitor connected in parallel with variable resistors representing the ion channels in the membrane. The change in membrane potential ($V_m$) is dependent on
the total current through the ion channels ($I_{\text{ion}}$) and membrane capacitance ($C_m$):

$$\frac{dV_m}{dt} = -\frac{I_{\text{ion}}}{C_m}.$$  

(3.1)

The current through each class of ion channel is governed by gating variables that describe the sensitivity and time-dependence of the ion channel to voltage and other stimuli. The gating of voltage-dependent ion channels is typically represented using the Boltzmann equation (Eq. 2.1).

### 3.3 Previous ICC Models

The first model to explicitly represent ICC as a separate cell type was a phenomenological model by Aliev et al. (2000). The development of biophysical cell models of ICC is at a relatively nascent stage, with the first full biophysical ICC model created in 2006 (Youm et al., 2006). Two further biophysical ICC models (Corrias and Buist, 2008; Faville et al., 2009) and two PMU models (Faville et al., 2008; Means and Sneyd, 2010) have been published more recently. A primary focus of biophysical ICC models is to quantitatively understand the signalling pathways that give rise to slow wave activity. The following sections present each of these models and discusses their respective merits, as well as potential areas for improvement. Note that these models sometimes use different terminology to refer to the same ion channels. For the sake of simplicity, this thesis will use a common symbol to refer to each type of current where it is clear that the same ion channel is being modelled, and will also provide the symbol used by the original authors.

#### 3.3.1 Aliev Slow Wave Model

Aliev et al. (2000) developed the first model of slow wave activity to incorporate separate representations of ICC and SMC. The simulated ICC and SMC were arranged in a one-dimensional line in two layers representing the ICC-MY and longitudinal muscle of the small intestine. Parameters could be adjusted to alter physiological behaviour, such as frequency of oscillation and resistivity of membrane coupling between cells. The Aliev
model was nevertheless a phenomenological model based on the FitzHugh-Nagumo neuron model (FitzHugh, 1961; Nagumo et al., 1962), with dimensionless parameters that were scaled to the appropriate units.

The Aliev model was used in anatomically realistic multiscale models of GI electrical activity (Cheng et al., 2007; Lin et al., 2006; Pullan et al., 2004). However, it had become evident that a biophysically-based approach to modelling slow waves was necessary to take advantage of burgeoning discoveries about the electrophysiology of ICC and SMC.

### 3.3.2 Youm Slow Wave Model

The ICC model by Youm et al. (2006), partially based on cardiac cell models, was the first biophysical cell model to include the ion channels and intracellular Ca$^{2+}$ transients that were thought to contribute to slow wave activity. The model included four ion channels: an inward rectifier K$^{+}$ current ($I_{K1}$), the L-type Ca$^{2+}$ current ($I_{CaL}$), a voltage-dependent DHP-resistant current ($I_{VDDR}$), and a Ca$^{2+}$-activated autonomous inward current ($I_{AI}$) carried by K$^{+}$, Ca$^{2+}$ and Na$^{+}$ ions. Three ion transporters were also included in the model: a NCX ($I_{NaCa}$), a Na$^{+}$/K$^{+}$ pump ($I_{NaK}$), and a PMCA pump ($I_{PMCA}$). Figure 3.1 depicts a schematic diagram of the model showing all the ion channels and transporters. The time-dependent membrane potential is described by the following expression,

$$-C_m \frac{dV_m}{dt} = I_{K1} + I_{CaL} + I_{VDDR} + I_{AI} + I_{NaCa} + I_{NaK} + I_{PMCA} + I_s,$$

(3.2)

where $C_m$ was set at 25 pF, the membrane capacitance of an ICC isolated from mouse small intestine (Koh et al., 2002; Youm et al., 2006), and $I_s$ is a stimulus current. Figure 3.4A shows slow wave activity simulated using the Youm et al. (2006) model.

The intracellular Ca$^{2+}$ transient in the ICC model by Youm et al. (2006) is governed by four Ca$^{2+}$ fluxes: an SR (or ER) uptake current, $I_{up}$; IP$_3$-mediated Ca$^{2+}$ release from the SR, $I_{IPR}$; a diffusive Ca$^{2+}$ leak current from the SR, $I_{leak}$; and the net sum of $I_{CaL}$, $I_{AI}$, $I_{VDDR}$, $I_{PMCA}$, and $I_{NaCa}$. The metabolism of IP$_3$ was governed by a three-state model of IP$_3$, IP$_4$, and PIP$_2$ production and degradation, with voltage- and Ca$^{2+}$-dependent rate constants. The synthesis of IP$_3$ controls the conductance of $I_{IPR}$. The SR was modelled...
3.3. Previous ICC Models

with separate sites for Ca\(^{2+}\) uptake from and release to the cytoplasm. SR Ca\(^{2+}\) uptake is governed by three currents: \(I_{\text{up}}\); \(I_{\text{leak}}\); and \(I_{\text{tr}}\), a transfer current between the uptake and release sites on the SR. Ca\(^{2+}\) release at the SR release site is governed by \(I_{\text{tr}}\) and \(I_{\text{IPR}}\). The time-dependent intracellular Ca\(^{2+}\) transient is described by the following expression,

\[
-z_{\text{Ca}} F V_c \frac{dCa_i}{dt} = I_{\text{CaL}} + I_{\text{VDDR}} + I_{\text{AI(Ca)}} - 2I_{\text{NaCa}} + I_{\text{PMCA}} - I_{\text{IPR}} + I_{\text{up}} - I_{\text{leak}},
\]

where \(z_{\text{Ca}}\) denotes the valence of Ca\(^{2+}\), \(F\) is the Faraday constant, \(V_c\) denotes the cell volume (712.5 \(\mu\)m\(^3\)), and \(I_{\text{AI(Ca)}}\) is the Ca\(^{2+}\) component of \(I_{\text{AI}}\) (Youn et al., 2006).

Figure 3.1: Schematic of the Youm et al. (2006) ICC model as implemented in CellML (Yu, 2010). The plasma membrane contains seven ion channels and transporters: \(I_{K1}\), \(I_{\text{CaL}}\), \(I_{\text{VDDR}}\), \(I_{\text{AI}}\), \(I_{\text{NaCa}}\), \(I_{\text{NaK}}\), and \(I_{\text{PMCA}}\), as described in the text. The sarcoplasmic reticulum Ca\(^{2+}\) store was separated into Ca\(^{2+}\) uptake and release regions (NSR and JSR, respectively).
3.3.3 Corrias and Buist Slow Wave Model

The ICC model by Corrias and Buist (2008) was principally based on the NSCC Hypothesis described in Section 2.9.1. The model ICC contained a single PMU with ER, mitochondria, and a small cytosolic subspace, representing the aggregate of all the PMUs in an ICC, as shown in Figure 3.2. A Ca$^{2+}$-inhibited NSC channel in the PMU membrane generated current, $I_{NSCC}$, in response to Ca$^{2+}$ cycling in the PMU. Because the PMUs were represented by a single bulk PMU, the simulated slow wave plateau phase was not generated by unitary potential summation, but by whole cell current flow through several ion channels, particularly a Ca$^{2+}$-activated Cl$^{-}$ channel, $I_{Cl}$.

The model included nine types of ion channels and one ion transporter in the bulk cytoplasm: $I_{VDDR}$, $I_{CaL}$ (called $I_{L\text{-type}}$ in the original literature), the delayed-rectifier $K_{V1.1}$ current ($I_{Kv1.1}$, originally called $I_{kv11}$), the ERG $K^{+}$ current ($I_{ERG}$), a Ca$^{2+}$-activated $K^{+}$ conductance ($I_{BK}$), a background $K^{+}$ leak current ($I_{K(B)}$, originally called $I_{Kb}$), the voltage-dependent Na$^{+}$ current ($I_{Nav1.5}$, originally called $I_{Na}$), $I_{NSCC}$, $I_{Cl}$, and a Ca$^{2+}$ extrusion mechanism representing the action of PMCA and NCX transporters ($I_{Ca(Ext)}$, called $I_{Ca\text{-EXT}}$ in the original literature). Figure 3.4B shows slow wave activity simulated using the Corrias and Buist ICC model. The time-dependent membrane potential is described by the following expression,

$$-C_m \frac{dV_m}{dt} = I_{VDDR} + I_{CaL} + I_{Kv1.1} + I_{ERG} + I_{BK} + I_{K(B)} + I_{Nav1.5} + I_{NSCC} + I_{Cl} + I_{Ca(Ext)}. \quad (3.4)$$

Corrias and Buist (2008) adapted an extensive description of intracellular Ca$^{2+}$ dynamics from Fall and Keizer (2001) to represent Ca$^{2+}$ handling in the PMU. Ca$^{2+}$ flux between the PMU cytosolic subspace and the greater cytoplasm is governed by a passive diffusive current ($J_{\text{leak}}$). The Ca$^{2+}$ handling dynamics of the mitochondria and ER induce Ca$^{2+}$ oscillations in the cytosolic subspace, from which Ca$^{2+}$ diffuses into the bulk cytoplasm through $J_{\text{leak}}$. This leads to a global increase in [Ca$^{2+}$], which in turn activates the Ca$^{2+}$-dependent ion conductances in the cell model: $I_{CaL}$, $I_{BK}$, and $I_{Cl}$. Conversely, $I_{NSCC}$ is activated by the falling phase of the Ca$^{2+}$ oscillations within the PMU. The time-dependent
intracellular Ca\(^{2+}\) transient is described by the following expression,

\[
-\frac{dC_{a_i}}{dt} = f_c \left( \frac{I_{CaL} + I_{VDDR}}{FV_c} + J_{\text{leak}} - J_{\text{Ca(Ext)}} \right),
\]  

where \(f_c\) denotes the cytosolic free Ca\(^{2+}\) proportion (set to 0.01), \(F\) denotes the Faraday constant, and \(V_c\) is the cytosolic volume (700\(\mu\)m\(^3\)).

![Diagram of the Corrias and Buist (2008) ICC model](image)

Figure 3.2: Schematic of the Corrias and Buist (2008) ICC model as implemented in CellML (Lloyd, 2010b). The ion channels illustrated in the plasma membrane are \(I_{\text{VDDR}}\), \(I_{L}\)-type, \(I_{K\text{v}1.1}\), \(I_{\text{ERG}}\), \(I_{\text{BK}}\), \(I_{\text{Na}}\), \(I_{\text{NSCC}}\), and \(I_{\text{Cl}}\), as described in the text. A Ca\(^{2+}\) extrusion pump is also shown. The PMU incorporating the NSCC is shown as a submembrane space (SS) between the mitochondria and ER.

### 3.3.4 Faville Unitary Potential and Slow Wave Models

The Faville ICC model, like the Corrias and Buist (2008) ICC model, was based on the NSCC Hypothesis described in Section 2.9.1. The Faville cell model is made from two distinct components, a PMU model (Faville et al., 2008) and a bulk cytoplasm model (Faville et al., 2009), and is the only biophysical model to incorporate multiple PMUs.
Faville et al. (2008) modelled the PMU using a compartmentalised approach. The PMU intracellular space was further divided into four subspaces: the ER, the mitochondria, the main cytoplasmic subspace (S1), and a smaller cytoplasmic subspace between the ER and mitochondria (S2), as shown in Figure 3.3.

The current across the PMU plasma membrane as implemented in the Faville et al. (2009) ICC model is the sum of current through three ion channels: an inward Ca\(^{2+}\) leak current (I\(_{\text{Ca}}\)), an inward Na\(^{+}\) leak current (I\(_{\text{Na}}\)), and a Ca\(^{2+}\)-inhibited NSC current (I\(_{\text{NSCC}}\)); and two ion transporters: a PMCA (I\(_{\text{PMCA}}\), originally called I\(_{\text{PM}}\)) and an outward Na\(^{+}\) pump (I\(_{\text{NaP}}\)). Figure 3.4C shows a unitary potential simulated using the Faville PMU model. The time-dependent unitary potential is described by the following expression,

\[
-C_m\frac{dV_{m(\text{PU})}}{dt} = I_{\text{ion(PU)}} = I_{\text{Ca}} + I_{\text{PMCA}} + I_{\text{NSCC}} + I_{\text{Na}} + I_{\text{NaP}}.
\]  (3.6)

Faville et al. (2008) adapted an IP\(_3\) model by Sneyd et al. (2000) to represent the Ca\(^{2+}\) release from the ER into the cytoplasm (J\(_{\text{IPR}}\)). In addition, four more intracellular Ca\(^{2+}\) fluxes were also defined: a SERCA pump (J\(_{\text{SERCA}}\)), a mitochondrial Ca\(^{2+}\) uniporter (J\(_{\text{MCU}}\)), a mNCX (J\(_{\text{NCX}}\)), and an inter-cytosolic subspace Ca\(^{2+}\) flux (J\(_{\text{S1S2}}\)). These ion channels and transporters govern the changes in [Ca\(^{2+}\)] in each of the four PMU subspaces. Specifically, the Ca\(^{2+}\) transient in S1 is governed by J\(_{\text{S1S2}}\), J\(_{\text{NCX}}\), I\(_{\text{Ca}}\), J\(_{\text{SERCA}}\); the Ca\(^{2+}\) transient in S2 is governed by J\(_{\text{IPR}}\), J\(_{\text{S1S2}}\), J\(_{\text{MCU}}\); the Ca\(^{2+}\) transient in the ER is governed by J\(_{\text{SERCA}}\) and J\(_{\text{IPR}}\); and the Ca\(^{2+}\) transient in the mitochondria is governed by J\(_{\text{MCU}}\) and J\(_{\text{NCX}}\).

The full ICC model by Faville et al. (2009) shown in Figure 3.3 contains multiple PMUs along with five ion channels in the bulk cytoplasm membrane, including the DHP-resistant T-type Ca\(^{2+}\) current (I\(_{\text{VDDR}}\), originally called I\(_{\text{Ca(T)}}\)), I\(_{\text{ERG}}\) (originally called I\(_{\text{K(ERG)}}\)), I\(_{\text{Kv1.1}}\) (originally called I\(_{\text{K(v1.1)}}\)), I\(_{\text{K(B)}}\), a small non-selective inward leak current (I\(_{\text{L}}\)), and a Ca\(^{2+}\) extrusion pump (I\(_{\text{Ca(Ext)}}\)). Using ten PMUs, Faville et al. (2009) demonstrated intracellular entrainment of unitary potentials and simulated intestinal slow waves at 17.4 cycles per
3.3. Previous ICC Models

minute (cpm) (Figure 3.4D). The time-dependent membrane potential is described by

\[-C_m \frac{dV_m}{dt} = I_{VDDR} + I_{\text{Ca(Ext)}} + I_{\text{ERG}} + I_{Kv1.1} + I_{K(B)} + I_L + \sum_{i=1}^{n_{\text{PU}}} I_{\text{ion(PU)}}, \]  

(3.7)

where $I_{\text{ion(PU)}}$ is the net current from one PMU, and $n_{\text{PU}}$ is the number of PMUs in the model.

3.3.5 Means and Sneyd Calcium Model

Unlike the models described above, the Means and Sneyd (2010) model did not simulate membrane potential and instead focused on Ca$^{2+}$ dynamics within the PMU. Means and Sneyd (2010) used the finite element method to create a spatial model of Ca$^{2+}$ handling in order to test the assumptions underlying the NSCC Hypothesis. [Ca$^{2+}$]$_i$ varied spatially within the PMU cytosol, the ER in the PMU, and a region of homogenised bulk cytosol and ER surrounding the PMU, enabling detailed analysis of Ca$^{2+}$ profiles in response to changes in Ca$^{2+}$ fluxes. As in the Faville et al. (2009) and Corrias and Buist (2008) ICC models, the PMU Ca$^{2+}$ depended on IP$_3$Rs and SERCA pumps in the ER, uniporters and mNCX on mitochondria, and PMCA and an inward Ca$^{2+}$ leak across the plasma membrane.

As outlined in Section 2.9.2, Means and Sneyd (2010) demonstrated that simulated PMUs cannot generate a [Ca$^{2+}$]$_i$ profile capable of causing Ca$^{2+}$-inhibited NSC channels to produce unitary potentials, thus providing strong evidence against the NSCC Hypothesis. In particular, maintaining the PMU [Ca$^{2+}$] high enough to inactivate the NSCC observed by Sanders et al. (2006) caused [Ca$^{2+}$]$_i$ in the bulk cytosol to rise to toxic concentrations (Means and Sneyd, 2010). It is worth noting that the Corrias and Buist (2008) and Faville et al. (2008) models implemented the NSCC Hypothesis using much lower $K_d$ values for NSCC inactivation (74.5 nM and 120 nM, respectively) than the 375 nM $K_d$ observed experimentally by Sanders et al. (2006).
Figure 3.3: Schematic of the Faville et al. (2009) ICC model as implemented in CellML (Lloyd, 2010a). The complete cell model incorporated multiple PMUs, an example of which is illustrated above the cell diagram. Each PMU contained four subspaces with separate Ca^{2+} concentrations: ER, mitochondria, cytosolic subspace 1, and cytosolic subspace 2. The ion channels and transporters in the PMU plasma membrane were: $I_{Ca}$, $I_{Na}$, $I_{NSCC}$, $I_{PM}$, and $I_{NaP}$, as described in the text. The ion channels and transporters included in the plasma membrane of the bulk cytoplasm model were: $I_{Ca(T)}$, $I_{K(ERG)}$, $I_{K(v1.1)}$, $I_{K(B)}$, $I_L$, and $I_{Ca(Ext)}$. 
3.3. Previous ICC Models

Figure 3.4: Simulated slow wave activity from previously published ICC models, simulated using CellML. (A) The Youm et al. (2006) ICC model of mouse small intestine slow waves at 31 cpm. (B) The Corrias and Buist (2008) ICC model of gastric slow waves. (C) The Faville et al. (2008) pacemaker unit model of unitary potential activity at gastric frequency. (D) The Faville et al. (2009) ICC model of mouse small intestine slow waves at 17 cpm with a unitary potential basis.

3.3.6 Comparison of the ICC Models

The three biophysical ICC models by Youm et al. (2006); Corrias and Buist (2008) and Faville et al. (2009) are compartmental models, but differ in their approach to modelling the intracellular Ca\(^{2+}\) transient and slow wave activity. Faville et al. (2008, 2009) implemented unitary potential summation using multiple PMUs, giving the model 41 subspaces in total (four per PMU plus one bulk cytoplasm), with a simple model of Ca\(^{2+}\) cycling between the ER and mitochondria. On the other hand, Corrias and Buist (2008) modelled bulk slow wave activity using a single PMU, including a complex model of mitochondrial function (Fall and Keizer, 2001), giving the ICC model four subspaces. Youm et al. (2006) did not consider the role of mitochondria in intracellular Ca\(^{2+}\) handling, so their model contained just the ER and the bulk cytoplasm.

More than half the ion channels used in the Corrias and Buist (2008) and Faville et al.
3. PREVIOUS MODELS OF ICC ACTIVITY

(2009) ICC models were common to both models. In contrast, the Youm et al. (2006) ICC model included a set of different ion channels, with only the voltage-gated Ca$^{2+}$ channels ($I_{VDDR}$ and $I_{CaL}$) overlapping with the other models, as detailed in Table 3.1. Youm et al. (2006) did include a NSC current ($I_{AI}$), but it was Ca$^{2+}$-activated, unlike the Ca$^{2+}$-inhibited $I_{NSCC}$ used by Faville et al. (2009) and Corrias and Buist (2008). The major omissions from the Faville ICC model were the Ca$^{2+}$-activited $I_{Cl}$, which has since been identified to have a critical role in the generation of slow wave activity (Hwang et al., 2009; Zhu et al., 2009), and the voltage-dependent Na$^+$ channel ($I_{Nav1.5}$), which is now believed to play an important role in mechanotransduction (Beyder et al., 2010) and GI pathologies (Saito et al., 2009).

Table 3.1: The types of plasma membrane ion channels and transporters included in the three biophysical models of ICC slow wave activity.

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<td><strong>NSC Channels:</strong></td>
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<tr>
<td>$I_{AI}$</td>
<td>Y</td>
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<td>$I_{NSCC}$</td>
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<td>Y</td>
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<tr>
<td>$I_{L}$</td>
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<td>Y</td>
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<tr>
<td><strong>Ca$^{2+}$ Channels:</strong></td>
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<tr>
<td>$I_{VDDR}$</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
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<tr>
<td>$I_{CaL}$</td>
<td>Y</td>
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<tr>
<td>$I_{Ca}$</td>
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<td><strong>Na$^+$ Channels:</strong></td>
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<tr>
<td>$I_{Nav1.5}$</td>
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<td>Y</td>
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<tr>
<td>$I_{Na}$</td>
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<td><strong>Cl$^-$ Channels:</strong></td>
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<tr>
<td>$I_{Cl}$</td>
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<td>Y</td>
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<td><strong>K$^+$ Channels:</strong></td>
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<tr>
<td>$I_{K1}$</td>
<td>Y</td>
<td></td>
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<tr>
<td>$I_{BK}$</td>
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<td></td>
<td>Y</td>
</tr>
<tr>
<td>$I_{Kv1.1}$</td>
<td></td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>$I_{ERG}$</td>
<td></td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>$I_{K(B)}$</td>
<td></td>
<td></td>
<td>Y</td>
</tr>
<tr>
<td><strong>Ion Transporters:</strong></td>
<td></td>
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<td></td>
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<tr>
<td>$I_{PMCA}$</td>
<td>Y</td>
<td></td>
<td>Y</td>
</tr>
<tr>
<td>$I_{Ca(Ext)}$</td>
<td></td>
<td></td>
<td>Y</td>
</tr>
<tr>
<td>$I_{NaK}$</td>
<td>Y</td>
<td></td>
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<tr>
<td>$I_{NaCa}$</td>
<td>Y</td>
<td></td>
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<tr>
<td>$I_{NaP}$</td>
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<td>Y</td>
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Computational efficiency of cell models is an important consideration, especially when incorporating cell models into large-scale simulations of electrophysiology (Du et al., 2010b). The slow wave outputs and computational efficiency in the three biophysical ICC models were simulated using CellML (Lloyd et al., 2008) and compared in Table 3.2. The ICC models by Faville et al. (2009) and Youm et al. (2006) both produced electrical activity in the frequency range of mouse small intestine slow waves, whereas the ICC model by Corrias and Buist (2008) produced gastric slow wave activity with a lower amplitude. In terms of computational efficiency, the ICC model by Faville et al. (2009) took the longest time to solve when the same solution method was used to solve each model, which can be attributed to the large number of subspaces and parameters in the Faville ICC model.

Table 3.2: Comparison of the outputs and efficiency of the three biophysical models of ICC slow wave activity. A 60 s period of slow wave activity was simulated on an Intel Core (T7800) using a forward Euler method with a fixed time step of 0.01 ms.

<table>
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<tr>
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<tbody>
<tr>
<td>RMP (mV)</td>
<td>−92</td>
<td>−68</td>
<td>−67</td>
</tr>
<tr>
<td>Peak $V_{m}$ (mV)</td>
<td>−2</td>
<td>−24</td>
<td>−2</td>
</tr>
<tr>
<td>Frequency (cpm)</td>
<td>31</td>
<td>3</td>
<td>17</td>
</tr>
<tr>
<td>Number of ODEs</td>
<td>14</td>
<td>22</td>
<td>78</td>
</tr>
<tr>
<td>Number of parameters</td>
<td>53</td>
<td>116</td>
<td>640</td>
</tr>
<tr>
<td>Simulation time (s)</td>
<td>17.8</td>
<td>22.7</td>
<td>83.9</td>
</tr>
</tbody>
</table>

The pacemaker mechanism underlying both the Faville and the Corrias and Buist ICC models is now in question, as described in Section 2.9.2 (Hwang et al., 2009; Means and Sneyd, 2010; Zhu et al., 2009). Nevertheless, both models still hold merit due to their respective methods of implementation. The Faville et al. (2009) model is unique in its use of multiple intracellular compartments to simulate the unitary potentials that have been hypothesised to give rise to whole cell slow wave activity. Additionally, the separation of the PMU cytosol into two subspaces is consistent with evidence that mitochondrial uniporters are highly localised within IP$_3$R clusters in guinea-pig colon SMC (Olson et al., 2010). On the other hand, Corrias and Buist (2008) developed a simpler model suitable for use in multiscale modelling applications, and entrainment of ICC slow wave activity has been modelled in two independent studies using the Corrias and Buist (2008) ICC model.
Applications of the Corrias and Buist Model

Two approaches were taken to model slow wave entrainment using the Corrias and Buist (2008) ICC model, both of which aimed to introduce a voltage-dependent component to the PMU Ca$^{2+}$ transient (Buist et al., 2010; Du et al., 2010c).

Du et al. (2010c) adapted an existing model of a voltage-dependent IP$_3$ synthesis pathway from Imtiaz et al. (2002) and incorporated it into the Corrias and Buist (2008) ICC model. Voltage-dependent IP$_3$ synthesis leads to release of intracellular Ca$^{2+}$ in response to changes in membrane potential. Thus the depolarisation of one model ICC entrains the slow wave activity of neighbouring ICC. The voltage-dependent IP$_3$ mechanism is described by

$$\frac{d[IP_3]}{dt} = \beta - \epsilon [IP_3] - V_{M4} \frac{[IP_3]^4}{K_4^4 + [IP_3]^4} + P_{MV} \left( 1 - \frac{V_m^8}{K_v^8 + V_m^8} \right),$$

(3.8)

where $\beta$ denotes IP$_3$ production in response to a chemical stimulus agent like acetyl choline, $\epsilon$ is the rate constant for linear IP$_3$ degradation, $V_{M4}$ is the rate constant for nonlinear IP$_3$ degradation, $P_{MV}$ denotes the maximal rate of voltage-dependent IP$_3$ synthesis, and $K_4$ and $K_V$ are the half-saturation constants for nonlinear IP$_3$ degradation and voltage-dependent IP$_3$ synthesis, respectively.

The other entrainment modelling study was conducted by Buist et al. (2010), who incorporated an additional I$_{VDDR}$ (denoted I$_{VDDRPU}$) into the PMU to directly couple [Ca$^{2+}$]$_{PMU}$ to membrane potential, along with a Ca$^{2+}$ extrusion current to maintain homeostasis. The expression for I$_{VDDRPU}$ is as follows,

$$I_{VDDRPU} = G_{VDDR} \cdot d_{PU} \cdot d_{VDDR} \cdot f_{VDDR} \left( V_m - E_{CaPU} \right),$$

(3.9)

where $G_{VDDR}d_{PU}$ is the maximum conductance of I$_{VDDRPU}$, $d_{VDDR}$ and $f_{VDDR}$ are the activation gate and inactivation gating variables, respectively, and $E_{CaPU}$ is the Nernst potential of Ca$^{2+}$ in the PMU. I$_{VDDRPU}$ added a voltage-dependent pathway for modulating [Ca$^{2+}$]$_{PMU}$ to the original Corrias and Buist (2008) ICC model, enabling entrainment of the pacemaker mechanism.
Du et al. (2010c) showed that their updated ICC model could be entrained by a single stimulus current occurring 5 s before the intrinsic onset of the slow wave, and coupled ICC were entrained with a phase lag of 3.1 s. Conversely, the Buist et al. (2010) ICC model could only be entrained by a stimulus current occurring less than 3 s before the intrinsic onset of the slow wave, and entrainment of coupled ICC occurred without a phase lag. The different responses of these two models may result from differences in the applied stimuli and coupling methods, or may be due to intrinsic differences in the entrainment methods used. Both updated ICC models were able to simulate slow wave propagation on a tissue scale (Buist et al., 2010; Du et al., 2010c). Even though the two approaches implemented were different, the common concept of perturbing Ca\textsuperscript{2+} concentration in the PMU to achieve entrainment is noteworthy.

### 3.4 The Need for a New Model

Chapter 2 considered the existing state of knowledge of ICC physiology, including a discussion of recent controversies and discoveries (Section 2.9.2). It is evident that the updated view of ICC pacemaker mechanisms in Section 2.9.4 also paves the way for an updated ICC model.

The existing biophysically-based models of ICC slow wave activity were completed prior to the discovery of Ano1 in ICC, so none of these models incorporated the Ano1 channel (Corrias and Buist, 2008; Faville et al., 2009; Gomez-Pinilla et al., 2009; Youm et al., 2006). Ano1 has since been shown to be crucial for slow wave activity (Hwang et al., 2009), and is likely to act as a pacemaker channel (Lees-Green et al., 2011b). Although the Corrias and Buist model did incorporate a Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} channel (Corrias and Buist, 2008), the channel model was not constructed using electrophysiological data from Ano1 currents so it did not capture the complex behaviour of Ano1.

The rise of Ano1 as a candidate pacemaker channel caused uncertainty about the role of NSC channels in ICC (Hwang et al., 2009; Lees-Green et al., 2011b; Zhu et al., 2009). Meanwhile, the detailed model of Ca\textsuperscript{2+} dynamics by Means and Sneyd (2010) cast further doubt on the plausibility of the NSCC Hypothesis on which the Corrias and Buist (2008)
and Faville et al. (2008, 2009) models were based. In addition, SOCE was not represented in any of the existing ICC models.

It is important to verify how the Ano1 channel may fit into a model of ICC pacemaker activity. Therefore, a key aspect of this thesis was the development of a new model of ICC slow wave activity that incorporates Ano1 as a pacemaker channel. Chapter 5 describes the development of an Ano1 channel model, and Chapter 6 describes the development of a novel ICC model.

On a different note, the existing models focus on the intrinsic pacemaker activity of ICC, and do not account for regulation of ICC activity by mechanoelectrical feedback (as introduced in Section 2.2.5). Therefore, Chapter 4 presents a model of ICC regulation by the mechanosensitive Nav1.5 channel.
Chapter 4

Sodium Channel Mechanosensitivity in ICC

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Mechanosensitivity helps regulate GI motility in response to the ever-changing contents of the GI tract. Mechanical stretch of smooth muscle tissue causes depolarisation and an increase in the frequency of slow waves, and these effects are partially mediated by ICC (Strege et al., 2003b; Won et al., 2005), although different types of mechanical stimuli—such as distension of the GI tract or hypoosmotic cell swelling—can have different effects on electrical activity (Wang et al., 2010d).

Mechanical deformation of cell membranes affects electrical activity by altering the current passed by mechanosensitive ion channels. Multiple cellular components of the GI tract contain mechanosensitive ion channels, including SMC (Lyford et al., 2002; Strege et al., 2003a; Wang et al., 2010b), ICC (Park et al., 2005; Strege et al., 2003b) and the enteric nervous system (Grundy and Brookes, 2012). The existing cell models of GI slow wave activity described in Section 3.3 do not contain mechanosensing mechanisms (Corrias and Buist, 2007, 2008; Faville et al., 2009). However, mechanosensitive ion channels can be incorporated into existing slow wave models by substituting a stretch-dependent ion channel model in place of an existing channel.
This chapter focuses on the mechanosensitivity of the voltage-gated Na\(^+\) channel, \(\text{Na}_V1.5\), which was introduced in Section 2.6.2 (Beyder et al., 2010; Strege et al., 2003b). A model of \(\text{Na}_V1.5\) mechanosensitivity was incorporated into the Faville et al. (2009) ICC model to investigate the impact of mechanical stimuli on slow waves. We note that Section 3.4 explained the need for a new ICC model to test how Ano1 may function as a pacemaker channel. A model developed for that purpose is presented later in Chapter 6.

The modelling of \(\text{Na}_V1.5\) presented in the current chapter was completed prior to the development of the new ICC model in Chapter 6. We expect that the results of simulating \(\text{Na}_V1.5\) mechanosensitivity would not qualitatively differ if they were repeated in another ICC model, because the \(\text{Na}_V1.5\) channel model depends only on membrane potential and stretch but not on the intracellular Ca\(^{2+}\) cycle or the identity of the other ion currents in ICC. Despite the shortcomings of the existing ICC models in describing the Ca\(^{2+}\)-activated pacemaker current, the ability of existing models to generate realistic pacemaker potentials and reproduce the expected voltage-dependent response has been validated (Corrias et al., 2012; Faville et al., 2009). The application of cell models that have suitable macroscopic properties but lack precise descriptions of ion currents has similarly been demonstrated by repeated use of the Hodgkin-Huxley action potential model (Sangrey et al., 2004).

### 4.1 Mechanosensitivity of Ion Channels

Mechanosensitivity of ion channels arises from movement of parts of the ion channel within the plasma membrane. Mechanosensitive ion channels can open or close in direct response to mechanical deformation of the plasma membrane or due to movement of the cytoskeleton (Beyder et al., 2013; Grundy and Brookes, 2012; Strege et al., 2003a). The voltage-dependence of mechanosensitive voltage-gated ion channels changes in response to mechanical input, thus helping regulate mechanoelectrical feedback loops by altering electrical output.

Many ion channels in the GI tract exhibit mechanosensitivity, including BK channels (Wang et al., 2010b), L-type Ca\(^{2+}\) channels (Lyford et al., 2002), volume-activated Cl\(^-\) channels (Park et al., 2005) and \(\text{Na}_V1.5\) channels (Strege et al., 2003b). In addition,
mechanosensitivity of Ano1 Cl\(^{-}\) channels was recently demonstrated in the bile duct (Dutta et al., 2013).

## 4.2 The Roles of Na\(_V\)1.5 in ICC and SMC

The Na\(_V\)1.5 channel, encoded by SCN5A, is a mechanosensitive Na\(^{+}\) channel expressed in ICC and SMC of the human small intestine (Ou et al., 2002; Strege et al., 2003b). Na\(_V\)1.5 currents inactivate quickly, and Na\(_V\)1.5 channels are thought to contribute to the upstroke phase of slow waves in ICC (Strege et al., 2003b) and the RMP in SMC (Holm et al., 2002). The Na\(_V\)1.5 inhibitors lidocaine and QX-314 decrease the upstroke rate of slow waves in human jejunum circular muscle strips, and also reduce the duration and frequency of slow waves. These observations support the theory that Na\(_V\)1.5 contributes to the upstroke phase and show that the channel is also active during repolarisation and RMP (Strege et al., 2003b). Patients with Na\(_V\)1.5 mutations have an increased prevalence of GI pathologies, indicating the functional relevance of Na\(_V\)1.5 to normal GI motility (Locke et al., 2006; Saito et al., 2009).

Na\(_V\)1.5 channels exhibit mechanosensitive properties, so they are proposed to play a role in sensing and responding to stretch in ICC and SMC (Strege et al., 2003a,b). The activation of Na\(^{+}\) currents in ICC from human small intestine was enhanced by using perfusion to induce shear stress, and in human circular smooth muscle strips, the frequency of slow waves increased when the tissue was stretched. Conversely, inhibition of the Na\(^{+}\) channel with lidocaine and QX-314 decreased slow wave frequency, slowed the rise rate and hyperpolarised RMP, and removal of Na\(^{+}\) ions from the bath resulted in complete loss of slow wave activity within several minutes (Strege et al., 2003b). These results together suggest that Na\(_V\)1.5 channels contribute to mechano-regulation of slow waves generated by ICC.

Beyder et al. (2010) subsequently investigated the response of Na\(_V\)1.5 channels expressed in HEK 293 cells to changes in pressure in the membrane patch during cell-attached patch clamp. Increased membrane tension due to either patch pressure or perfusion-induced shear stress caused an increase in peak Na\(^{+}\) current and accelerated kinetics of activation,
as shown in Figure 4.1A (Beyder et al., 2010; Strege et al., 2003a). Patch pressure also accelerated fast inactivation (Figure 4.1A) and slowed recovery from inactivation, whereas inactivation kinetics were unaffected by shear stress. Pressure-induced changes in Na$^+$ current kinetics appeared to result from mechanosensitivity of the voltage sensors within the channels. The increase in Na$^+$ current due to patch pressure appeared to be due to an increase in the number of active channels in the patch, but it is unclear whether the same mechanism is responsible for increased peak current during shear stress (Beyder et al., 2010, 2013).

4.3 Sodium Channel Model

Beyder et al. (2010) quantified the changes in Na$_V$1.5 steady-state voltage-dependence and kinetics of activation and inactivation in response to mechanical patch pressure stimuli. This enabled a model of Na$_V$1.5 mechanosensitivity to be constructed and used to investigate the physiological impacts of changes on slow wave generation by ICC (Beyder et al., 2013; Lees-Green et al., 2011a).

The Na$^+$ channel activation and inactivation gating variables, $d_{Na}$ and $f_{Na}$, respectively, were calculated using sigmoidal Boltzmann equations (equations 4.1–4.5). Patch pressures of $-10$ to $-50$ mmHg shifted the half-activation and half-inactivation voltages by $0.7$ mV mmHg$^{-1}$, and consequently hyperpolarised the voltage range of the window current, as shown in Figure 4.1B–C (Beyder et al., 2010). These shifts in half-activation potentials were also able to account for the changes in the activation and inactivation kinetics, as shown in Table 4.1. A trend of steeper activation slopes with increased patch pressure was also observed. These quantitative changes, along with the voltage-dependence of the Na$^+$ current during control conditions, were used to develop the Na$^+$ channel model.

The gating variables, $d_{Na}$ and $f_{Na}$, were described by the following differential equations:

$$\frac{dd_{Na}}{dt} = \alpha_d (1 - d_{Na}) - \beta_d \cdot d_{Na}, \quad (4.1)$$

$$\frac{df_{Na}}{dt} = \alpha_f (1 - f_{Na}) - \beta_f \cdot f_{Na}. \quad (4.2)$$
Figure 4.1: Pressure dependence of NaV1.5 currents recorded from HEK 293 cells. A: NaV1.5 currents evoked by a step depolarisation from $-120\text{ mV}$ to $-10\text{ mV}$ at 0 mmHg and $-30\text{ mmHg}$ patch pressure. B, C: Voltage-dependence of NaV1.5 current activation (B) and inactivation (C) fitted with Boltzmann curves at patch pressures of 0 mmHg to $-50\text{ mmHg}$, showing a leftward shift in voltage-dependence with increased pressure. Inset: The overlap in voltage-dependence of activation and inactivation, which results in a window current, is shown at 0 mmHg (right) and $-40\text{ mmHg}$ (left), with a leftward shift in the window current with pressure. Reproduced from Beyder et al. (2010) with permission from John Wiley and Sons.
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\( \alpha \) and \( \beta \), the rate constants for channel opening and closing, respectively, were governed by the following equations for both activation (\( \alpha_d \) and \( \beta_d \)) and inactivation (\( \alpha_f \) and \( \beta_f \)):

\[
\alpha = \tau \left( \frac{1}{1 + e^{(V_m - V_h)/k}} \right), \tag{4.3}
\]

\[
\beta = \tau \left( 1 - \frac{1}{1 + e^{(V_m - V_h)/k}} \right), \tag{4.4}
\]

where \( V_h \) is the half-potential of (in)activation, \( k \) is the slope of (in)activation, and \( \tau \) is the (in)activation time constant in \( s^{-1} \). \( \tau \) is calculated as

\[
\tau = T_{a1} + \frac{T_{a2} - T_{a1}}{1 + e^{T_k(V_m-T_V)}}. \tag{4.5}
\]

\( V_h, k, \) and \( T_V \) are all variables of pressure, \( p \), in mmHg. The parameter values and pressure-dependent equations are listed in Table 4.1.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Activation</th>
<th>Inactivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>( V_h ) (mV)</td>
<td>(-36.5 + 0.72p )</td>
<td>(-75 + 0.68p )</td>
</tr>
<tr>
<td>( k ) (mV)</td>
<td>( 5.6 + 0.022p )</td>
<td>( 6.0 )</td>
</tr>
<tr>
<td>( T_{a1} ) (s(^{-1}))</td>
<td>( 100 )</td>
<td>( 110 )</td>
</tr>
<tr>
<td>( T_{a2} ) (s(^{-1}))</td>
<td>( 2070 )</td>
<td>( 2200 )</td>
</tr>
<tr>
<td>( T_V ) (mV)</td>
<td>(-20 + 0.72p )</td>
<td>(-12 + 0.68p )</td>
</tr>
<tr>
<td>( T_k ) (mV(^{-1}))</td>
<td>(-0.6 )</td>
<td>(-0.09 )</td>
</tr>
</tbody>
</table>

4.4 Verifying the Role of Na\(_V\)1.5 in the ICC Model

The mechanosensitive Na\(_V\)1.5 channel model was incorporated into the Faville et al. (2009) small intestine ICC model by replacing the cation leak current (referred to as \( I_L \) in Chapter 3) with \( I_{Na1.5} \) (Beyder et al., 2013; Lees-Green et al., 2011a):

\[
I_{Na1.5} = g_{Na} \cdot d_{Na} \cdot f_{Na} \cdot (V_m - E_{Na}), \tag{4.6}
\]
4.4. Verifying the Role of Na\textsubscript{V}1.5 in the ICC Model

Figure 4.2: Effect of inhibiting Na\textsubscript{V}1.5 channels in the Faville et al. (2009) ICC model of small intestine slow wave activity. Membrane potential is shown for simulations with maximum Na\textsubscript{V}1.5 conductance (1.6 nS), 50\% inhibition (0.8 nS), and complete block of Na\textsubscript{V}1.5 (0 nS).

where $g_{Na}$ is the maximum conductance, set at 1.6 nS, and $E_{Na}$ is the Nernst potential for Na\textsuperscript{+} ions.

Inhibition of the Na\textsuperscript{+} channel was simulated to validate the modified ICC model by comparing the contribution of the simulated Na\textsuperscript{+} current to the predicted role of Na\textsubscript{V}1.5 from experiments (Strege et al., 2003b), as detailed in Section 4.2. Half-maximal inhibition of $I_{Nav1.5}$ caused hyperpolarisation of RMP, decreased frequency and reduced upstroke rate (Figure 4.2, blue trace). Comparable results were seen in human jejunum smooth muscle strips when inhibiting Na\textsubscript{V}1.5 channels with lidocaine or QX-314 (Strege et al., 2003b).

Completely blocking $I_{Nav1.5}$ caused a hyperpolarisation of RMP and complete loss of slow waves, such that individual unitary potentials could be seen (Figure 4.2, green trace). Similarly, Strege et al. (2003b) showed that removal of Na\textsuperscript{+} ions from the bath resulted in complete loss of slow wave activity in smooth muscle strips within several minutes.
4.5 Modelling Stretch in ICC

Simulations were used to investigate the effects of mechano-regulation of Na\(^+\) current on the ICC slow wave. To simulate stretch using this model, negative pressures of \(-10\) mmHg to \(-60\) mmHg were applied to Na\(_V\)1.5 channel model, based on the pressures applied in the patch clamp experiments by Beyder et al. (2010), from which the model equations were developed. It should be noted that the relationship between pressure and membrane tension is non-linear (Beyder et al., 2013), and the pressure applied during cell-attached patch experiments may not be directly comparable to the tension experienced by ion channels under physiological conditions of stretch or contraction.

The largest pressure-induced changes in the simulated slow waves were observed at the highest pressure of \(-60\) mmHg (Figure 4.3, Table 4.2). Pressures of \(-30\) mmHg and below had negligible effect on the simulated slow waves.

Figure 4.3 shows that mechanical stimulation causes depolarisation of RMP, hyperpolarisation of the plateau phase, and an increased frequency. The pressure-dependent shift in the half-potentials of activation and inactivation caused a hyperpolarising shift in window current towards the RMP, thus increasing the baseline Na\(^+\) current and depolarising the RMP.

In addition, four characteristics of slow wave morphology were quantified: (i) RMP, (ii) rise rate during the upstroke phase, (iii) half-width (duration at half-maximal amplitude), and (iv) frequency, as shown in Table 4.2. Note that RMP was measured as the baseline potential prior to the slow wave upstroke; the most polarised potential occurs immediately after repolarisation and is not markedly changed by stretch, as seen in Figure 4.3. The largest applied pressure of \(-60\) mmHg caused the greatest quantitative changes, with smaller changes observed during \(-40\) mmHg pressure. The changes at \(-60\) mmHg included a 5\% depolarisation of RMP, an 11\% increase in rise rate, a 0.9\% increase in frequency, and a 5\% increase in the duration. The increased frequency and membrane depolarisation agree with the experimentally observed effects of stretch on GI smooth muscle tissue (Strege et al., 2003b; Won et al., 2005).

Although frequency increased by just 0.9\%, the increase of 0.15 min\(^{-1}\) with \(-60\) mmHg
pressure is similar to the change in frequency observed in strips of human jejunum, in which stretch increased slow wave frequency from $8.2 \pm 0.2$ to $8.50 \pm 0.15$ min$^{-1}$ ($P < 0.05$) (Strege et al., 2003b). Functionally, the combination of increased frequency and duration would act to increase the length of time during which contraction can occur, because the primary mechanism for SMC excitation-contraction coupling is voltage-dependent Ca$^{2+}$ influx during the slow wave plateau (Sanders, 2008). Depolarisation of RMP would increase the overall excitability of the smooth muscle tissue.

The pressures applied in the Na$\text{V}_{1.5}$ model cannot be quantitatively compared to the tension applied during tissue stretch. However, the simulation results confirm that Na$\text{V}_{1.5}$ mechanosensitivity is likely to contribute to the effects of stretch on slow waves in GI tissue. The effects of pressure on the simulated slow wave all suggest an excitatory role for Na$\text{V}_{1.5}$ channels during stretch, in agreement with the effects of stretch on sections of human small intestine tissue (Strege et al., 2003b).
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Table 4.2: Effect of stretch on slow wave characteristics. The quantitative changes in RMP, upstroke rate, half-width, and frequency are provided for 0 mmHg (resting), −40 mmHg and −60 mmHg conditions.

<table>
<thead>
<tr>
<th>Pressure</th>
<th>RMP (mV)</th>
<th>Rate of rise (mV s(^{-1}))</th>
<th>Half-width (s)</th>
<th>Frequency (min(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mmHg</td>
<td>−70.2</td>
<td>623</td>
<td>0.615</td>
<td>16.98</td>
</tr>
<tr>
<td>−40 mmHg</td>
<td>−68.1</td>
<td>669</td>
<td>0.626</td>
<td>17.04</td>
</tr>
<tr>
<td>−60 mmHg</td>
<td>−66.4</td>
<td>693</td>
<td>0.645</td>
<td>17.13</td>
</tr>
</tbody>
</table>

4.6 Future Directions

The model of Na\(_V\)1.5 mechanosensitivity described in this chapter included pressure-dependent changes in the half-potentials of steady-state activation and inactivation, the slope of the activation curve, and the time constants of activation and inactivation. In patch clamp experiments, pressure also caused the stabilisation of inactivated states and slowed recovery from inactivation (Beyder et al., 2010), but these effects could not be easily implemented in the Hodgkin-Huxley type ion channel model. These aspects of Na\(_V\)1.5 mechanosensitivity may contribute to the mechano-regulation of the repolarisation phase of slow waves, excitability, and RMP. An existing Markov formulation of Na\(_V\)1.5 (Poh et al., 2012) could be modified to model the effect of stretch on the inactivated states and the kinetic link between activation and inactivation.

An important goal of modelling stretch is to expand this work to investigate mechanosen-
sitivity on the tissue scale. The dynamic effects of mechanoelectrical and electromechanical feedback cycles on the tissue and organ level can be further explored by modelling, using both ICC and SMC models with integrated ion channel mechanosensitivity and electromechanical coupling (Du et al., 2011; Gajendiran and Buist, 2011).

Modelling mechanoelectrical feedback cycles will require mechanosensitive ion channel models to be a function of a relevant mechanical stimulus like tension. The Na\(_V\)1.5 channel model presented in this chapter is modulated as a function of patch pressure. The relationship between patch pressure applied \textit{in vitro} and tissue stretch experienced \textit{in vivo} will need to be characterised so that models of ion channel mechanosensitivity can be updated and validated to ensure a physiological response to mechanical stimuli.
In addition to the intrinsic mechanosensitive properties of NaV1.5 channels in both ICC and SMC, Na⁺ currents may also be linked to the Ca²⁺ dynamics that control SMC contraction. The NCX in cardiac myocytes links excitation-contraction coupling to Na⁺ dynamics, and nanodomains including voltage-dependent Na⁺ channels may be involved in regulating contraction (Aronsen et al., 2013). Furthermore, NaV1.5 channels are modulated by Ca²⁺, calmodulin, and Ca²⁺/calmodulin-dependent protein kinase II (Wagner et al., 2006; Wingo et al., 2004; Tan et al., 2002). A similar Na⁺–Ca²⁺ feedback loop may add another layer to the mechanoelectrical feedback loop by which NaV1.5 regulates contraction in SMC.

It is likely that most channels in the GI tract exhibit some mechanosensitivity. In general, any channel that has changes in its in-plane area during gating should be mechanically sensitive (Markin and Sachs, 2004). Many channels in the GI tract other than NaV1.5 have already been shown to be mechanosensitive, including L-type Ca²⁺ channels (Lyford et al., 2002) and BK channels (Wang et al., 2010b), but the level of available data, especially from native GI cells, is limited. Tissue level models will be enhanced by incorporating each of these ion channel models into both ICC and SMC, where appropriate.
Chapter 5

Anoctamin1 Ca$^{2+}$-Activated Cl$^{-}$ Channel Model


The Ca$^{2+}$-activated Cl$^{-}$ channel, Ano1, is a candidate pacemaker channel that is highly expressed in all classes of ICC in the human and murine gastrointestinal tracts (Gomez-Pinilla et al., 2009), as introduced in Section 2.6.3. Identifying the pacemaker channel that initiates slow waves is a crucial step in understanding pacemaker activity. Other proposed candidates for the pacemaker channel include Ca$^{2+}$-activated maxi Cl$^{-}$ channels (Wright et al., 2012); Ca$^{2+}$-inhibited NSC channels (Koh et al., 2002); Ca$^{2+}$-activated NSC channels (Goto et al., 2004); or TRPM7 NSC channels (Kim et al., 2005). However, the evidence for Ano1’s role as a pacemaker channel is particularly strong, because Ano1 knockout mice do not exhibit slow waves and have significantly impaired smooth muscle contraction, indicating that Ano1 is essential for initiating the electrical pacemaker activity of ICC (Huang et al., 2009; Hwang et al., 2009).

Ano1 channels have a complex dependence on Ca$^{2+}$ and voltage. They are slowly activating and outwardly rectifying at low [Ca$^{2+}$]$_i$, but at high [Ca$^{2+}$]$_i$ they activate rapidly and have a linear voltage-current relationship (Xiao et al., 2011). Ca$^{2+}$-activated Cl$^{-}$ currents were studied extensively prior to the discovery that the anoctamin family of
proteins, particularly Ano1, are a major component of Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} channels (Britton et al., 2010; Tian et al., 2012). Mathematical models simulating these Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} currents were also published.

Arreola et al. (1996) developed a simple Markov model of Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} currents in rat parotid acinar cells. The model simulated channel activation reasonably well, but at high [Ca\textsuperscript{2+}] (1000 nM) the simulated activation kinetics were too slow and the simulated tail currents deactivated very rapidly (Arreola et al., 1996). Kuruma and Hartzell (2000) developed a more detailed Markov model based on Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} currents recorded from *Xenopus* oocytes. Using Monte-Carlo simulation, their model was able to reproduce many aspects of their experimental data, although the simulated rates of activation and deactivation were quantitatively different from those from experimental recordings. In particular, the time course of the tail currents was much slower in simulations than in experimental data (Kuruma and Hartzell, 2000).

The aim for this thesis was to find or develop a model that adequately reproduced the behaviour of Ano1 channels without introducing unnecessary complexity or computational expense. Although the existing models described above provided reasonable approximations to experimental data, quantitative differences are apparent in Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} currents recorded from different cell types (Arreola et al., 1996; Kuruma and Hartzell, 2000; Xiao et al., 2011), and we cannot ascertain whether the endogenous currents underlying previous models were Ano1 currents or whether other Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} channels or modulatory proteins were involved (e.g. Sagheddu et al., 2010). Developing a new model based on currents recorded from Ano1 channels in a heterologous expression system provides confidence that the model will capture the characteristics of Ano1 currents.

Furthermore, the models described above were multistate Markov models, which have increased complexity and reduced computational efficiency compared to Hodgkin-Huxley type ion channel models. Despite their greater level of detail, the ability of Markov models to simulate whole-cell ion currents more accurately than Hodgkin-Huxley models has not been conclusively shown (Fink and Noble, 2009), so our preference was to use a simple model. Britton et al. (2010) suggested a modified Boltzmann function could be used to model steady-state activation of Ca\textsuperscript{2+}-activated Cl\textsuperscript{−} currents. We attempted to
parameterise the modified Boltzmann equation using experimental recordings of Ano1 currents published by Xiao et al. (2011), but could not reproduce experimentally observed steady-state behaviour. Therefore, the decision was made to develop a new, simple model of Ano1 based on recordings of Ano1 currents heterologously expressed in HEK 293 cells (Lees-Green et al., 2012, 2014).

### 5.1 The Ano1 Model

The voltage- and Ca\(^{2+}\)-dependent behaviour of mouse Ano1 channels heterologously expressed in HEK 293 cells was recently characterised (Xiao et al., 2011). Equations were reported for both the steady-state Ca\(^{2+}\)-dependent activation as a function of voltage, and voltage-dependent activation as a function of \([\text{Ca}^{2+}]_i\). Steady-state voltage-dependent activation at a fixed Ca\(^{2+}\) concentration was described by a Boltzmann function:

\[
\frac{G}{G_{\text{max, Ca}}} = \frac{1}{1 + e^{(V_m - V_h)zF/RT}},
\]

(5.1)

where \(G/G_{\text{max, Ca}}\) is normalised conductance; \(V_h\) is the membrane potential at which \(G/G_{\text{max, Ca}}\) is half-maximal; and \(z\) is “the equivalent gating charge associated with voltage-dependent channel opening” (Xiao et al., 2011). \(V_h\) was shown to vary dramatically with changes in \([\text{Ca}^{2+}]_i\), but the relationship between \([\text{Ca}^{2+}]_i\) and \(V_h\) was not fully characterised due to experimental limitations on the range of membrane potentials that could be studied under voltage clamp.

The \([\text{Ca}^{2+}]_i\) at which steady-state Ca\(^{2+}\)-dependent activation was half-maximal, \(EC_{50}\), was voltage-dependent and the change in \(EC_{50}\) with voltage was described by the following expression:

\[
EC_{50} = EC_{50(0mV)}e^{-k_cV_m},
\]

(5.2)

where \(EC_{50(0mV)}\) is the value of \(EC_{50}\) at 0 mV, equal to 1.39 µM; and \(k_c\) is 0.01248 mV\(^{-1}\), as reported by Xiao et al. (2011).

The two equations above were used as the basis for a novel Hodgkin-Huxley type model of coupled voltage- and Ca\(^{2+}\)-dependent Ano1 activation. The steady-state activation for
an ion channel is given by $G/G_{\text{max}}$ (or $I/I_{\text{max}}$), whereas the equations previously published by Xiao et al. (2011) scaled $G$ to the maximal conductance recorded at a set value of $[\text{Ca}^{2+}]_i$ or $V_m$; $G_{\text{max,Ca}}$ or $G_{\text{max,V}}$ respectively. Thus, the steady-state open probability ($O_{\text{Ano1}}$) was modelled by scaling $G/G_{\text{max,V}}$ by the $G/G_{\text{max,Ca}}$ at a very high $[\text{Ca}^{2+}]_i$, giving $O_{\text{Ano1}}$ as the product of a Boltzmann function for voltage-dependent activation and a Hill equation for Ca$^{2+}$ binding:

$$O_{\text{Ano1}} = \frac{1}{(1 + e^{(V_h - V_m)k_v}) \left(1 + \left(\frac{\text{EC}_{50}}{\text{Ca}_{\text{Ano1}}}\right)^2\right)},$$

(5.3)

where $V_h$, the half-activation potential at high $[\text{Ca}^{2+}]_i$, was set at $-100$ mV to reproduce correct Ano1 activation profile; $k_v$, the slope factor associated with the gating charge, is $0.0156 \text{ mV}^{-1}$, as reported by Xiao et al. (2011); $\text{Ca}_{\text{Ano1}}$ is the $[\text{Ca}^{2+}]_i$ in the vicinity of the Ano1 channel; and $\text{EC}_{50}$ is calculated as in Equation 5.2. The steady-state open probability of the Ano1 channel model is shown in Figure 5.1.

Like steady-state activation, Ano1 activation kinetics are dependent on both $[\text{Ca}^{2+}]_i$ and
membrane potential. Xiao et al. (2011) quantified the time constants of Ca$^{2+}$-dependent activation, Ca$^{2+}$-dependent deactivation and tail current deactivation, as follows. During voltage clamp experiments, Ano1 current activated rapidly and deactivated very slowly in the presence of high [Ca$^{2+}$], whereas activation occurred slowly and deactivation occurred more rapidly in the presence of low [Ca$^{2+}$]. Fast perfusion of high [Ca$^{2+}$] solution also caused rapid Ano1 activation, and removal of high [Ca$^{2+}$] solution caused slow deactivation of Ano1 currents.

In order to develop an empirical description of the time constant for the Ano1 model, the published Ca$^{2+}$-dependent activation and deactivation rates were fitted by hand to a set of exponential functions. The complete set of equations describing the Ano1 model time constant are detailed in Appendix B.3.

### 5.2 Results

The Ano1 model was verified against published data at various Ca$^{2+}$ and voltage levels. The steady-state open probability of the Ano1 channel model in Figure 5.1 shows that open probability is increased by both Ca$_{Ano1}$ and membrane potential, which results in strong outward rectification of steady-state Ano1 current at lower Ca$^{2+}$ concentrations and a more linear current-voltage relationship at higher Ca$^{2+}$ concentrations, as expected.

The kinetics of the Ano1 model (Figures 5.2 and 5.3) were compared with published voltage-clamp and fast Ca$^{2+}$ perfusion experiments (Xiao et al., 2011). For these simulations Ano1 currents were calculated by setting a reversal potential of 0 mV and a maximal conductance of 0.5 nS.

In the voltage clamp simulations the voltage protocol stepped the Ano1 model from a holding potential of 0 mV to a $V_m$ between $-100$ and $+100$ mV in 40 mV increments for 800 ms, followed by a hyperpolarising step to $-100$ mV (inset in Figure 5.2A). Voltage clamp simulations were carried out at three different values of fixed [Ca$^{2+}$]: 0.1 µM (Figure 5.2A), 1 µM (Figure 5.2B), and 10 µM (Figure 5.2C). The simulated voltage-dependent activation qualitatively matches the experimentally observed behaviour, with higher Ca$^{2+}$ concentrations producing larger and more rapid activation (Figure 5.2D). However, at
Figure 5.2: Simulated voltage-clamp experiments to test the response of the Ano1 model to the voltage protocol used by Xiao et al. (2011). A–C: From a holding potential of 0 mV, the Ano1 model was stepped for 800 ms to a $V_m$ between $-100$ mV and $+100$ mV in 40 mV increments, followed by a hyperpolarising step to $-100$ mV, as shown in the inset. Depolarising steps activate Ano1 currents more strongly and rapidly at higher Ca$^{2+}$ concentrations, as expected. However, above 1 $\mu$M Ca$^{2+}$ the decay of the simulated tail currents following a hyperpolarising step occurs more rapidly than expected based on experimental observations. D: Ano1 currents recorded from HEK 293 cells using the same voltage protocol, for comparison with voltage-clamp simulations (Reprinted from Xiao et al., 2011).

Higher Ca$^{2+}$ concentrations the decay of the tail currents following a hyperpolarising step occurs much more rapidly in the simulations than in previously published data (Xiao et al., 2011), indicating that the model does not fully capture the time-dependence of voltage-dependent deactivation (Figure 5.2C).

Rapid perfusion experiments were performed by Xiao et al. (2011) in which [Ca$^{2+}$] was changed within several milliseconds. These experiments were reproduced using the Ano1 model by setting $V_m$ at a fixed holding potential (ranging from $-100$ to $+100$ mV in 40 mV increments) and applying a step change in $Ca_{Ano1}$ from 0.1 $\mu$M to 20 $\mu$M for 800 ms. The Ano1 model produces fast Ca$^{2+}$-dependent activation at all voltages and slower deactivation upon Ca$^{2+}$ removal with a strongly voltage-dependent deactivation...
rate (Figure 5.3), in qualitative agreement with published data (Xiao et al., 2011).

Figure 5.3: Top: Simulated rapid Ca\(^{2+}\) perfusion experiments, to compare the response of the Ano1 model to published rapid perfusion experiments (Xiao et al., 2011).\(V_m\) in the Ano1 model was set at a fixed holding potential ranging from −100 mV to +100 mV and Ca\(_{Ano1}\) was stepped from 0 µM to 20 µM for 800 ms. Ano1 activation upon application of 20 µM Ca\(^{2+}\) occurs quickly and the rate of activation is only weakly voltage-dependent, while deactivation upon removal of 20 µM Ca\(^{2+}\) is slow and the deactivation rate is strongly voltage-dependent. Bottom: Ano1 currents recorded from HEK 293 cells using the same rapid perfusion protocol, for comparison with rapid Ca\(^{2+}\) perfusion simulations (Reprinted from Xiao et al., 2011).

5.3 Discussion

This chapter presents a novel mathematical model of the Ano1 Ca\(^{2+}\)-activated Cl\(^-\) channel based on electrophysiological measurements of Ano1 currents. Although the model uses a
simpler formulation than previous models of Ca\(^{2+}\)-activated Cl\(^{-}\) channels (Arreola et al., 1996; Kuruma and Hartzell, 2000), it successfully reproduces the steady-state behaviour, activation kinetics, and Ca\(^{2+}\)-dependent inactivation kinetics of experimentally observed Ano1 currents (Britton et al., 2010; Hartzell et al., 2005; Xiao et al., 2011).

A limitation of the Ano1 model is its inability to reproduce the slow deactivation observed during voltage clamp experiments with higher Ca\(^{2+}\) concentrations. Adjusting the time constant parameters in an attempt to better simulate tail currents diminished the ability of the model to reproduce other aspects of Ano1 kinetics, particularly activation rates.

The same limitation was evident in previous models of Ca\(^{2+}\)-activated Cl\(^{-}\) channels too, in which tail currents either deactivated too slowly (Kuruma and Hartzell, 2000) or too fast (Arreola et al., 1996). The prevalence of difficulties in modelling Ano1 tail currents suggests that the usual approaches used to characterise and model ion channels may not be sufficient for Ano1 channels, which have more complex activation kinetics than traditional voltage- or ligand-gated channels (Schroeder et al., 2008; Xiao et al., 2011).

It is important to note that different variants of Ano1 generate quantitatively different currents. Multiple isoforms of Ano1 are produced by alternative splicing of four different exons, \(a\), \(b\), \(c\) and \(d\). The Ano1 model presented here is based on the properties of mouse Ano1\((a,c)\) expressed in HEK 293 cells (Xiao et al., 2011). The expression of alternative splice variants is known to alter the kinetics and Ca\(^{2+}\) sensitivity of the channel (O’Driscoll et al., 2011).

The Ano1\((a,c)\) variant used to develop the Ano1 model presented in this study may not be the most prevalent splice variant in ICC. Verifying which Ano1 splice variants are commonly expressed in human and mouse ICC and characterising the electrophysiological properties of these variants would allow more precise modelling of the role of Ano1 in ICC. Different Ano1 isoforms were shown to be expressed at different rates in human diabetic gastroparesis compared with non-diabetic controls, but there was also variable expression of Ano1 isoforms in the control subjects (Mazzone et al., 2011). The expression of human Ano1 variants is controlled by a recently identified promoter, which may impact on the properties of Ano1 currents (Mazzone et al., 2013), and different Ano1 variants can
interact, further modifying their behaviour (O’Driscoll et al., 2011). These factors may complicate attempts to determine which isoform or isoforms should be included in an ICC model. When expressed in HEK 293 cells, different Ano1 variants were shown to have different kinetics and current density (Mazzone et al., 2011). However, the extent to which expression of different splice variants alters the macroscopic behaviour of Ano1 channels in ICC is yet to be determined, and will require further experimental and modelling studies.
Chapter 6

ICC Model


This chapter describes the development of an ICC model consistent with the generalised pacemaker hypothesis put forward in Chapter 2. The ICC model incorporates the Ano1 model presented in Chapter 5 in order to investigate how the Ano1 channel fits into a model of pacemaker activity, and also includes SOCE as a key component of the Ca\(^{2+}\) dynamics (Lees-Green et al., 2014).

The primary aim of the model was to elucidate how Ano1 Ca\(^{2+}\)-activated Cl\(^{-}\) channels contribute as pacemaker channels in ICC. The precise function of Ano1 current in ICC depends on the Cl\(^{-}\) equilibrium potential (E\(_{\text{Cl}}\)). Measurements of intracellular Cl\(^{-}\) concentration ([Cl\(^{-}\)]\(_i\)) indicate that E\(_{\text{Cl}}\) in murine ICC-MY is \(-40\) to \(-50\) mV (Zhu et al., 2010), but microelectrode techniques used to record slow waves may lead to Cl\(^{-}\) loading (Blatt and Slayman, 1983), and previous studies have suggested that Cl\(^{-}\) currents reverse positive to the slow wave plateau (Kito and Suzuki, 2003). Therefore, the present study aimed to test the effect of varying E\(_{\text{Cl}}\) on slow waves in ICC-MY.

In addition, the identity of the ion channels active during the plateau phase remains unclear. Another aim was to predict the characteristics of the ion channels that are required to reproduce the expected slow wave morphology. Specifically, suitable ion channel candidates may be activated by voltage or Ca\(^{2+}\), remain active for the duration of the plateau phase, and carry an inward current at the plateau.
The specifics of the hypothesis underlying the model are outlined first in Section 6.1.1, followed by the details of the mathematical model itself in Sections 6.1.2–6.1.8. Then Section 6.2 reports the results of simulations testing the impact of altering $E_{Cl}$, which show that the ability to reproduce the characteristic slow wave morphology is highly dependent on $E_{Cl}$. The results of varying the ion currents in the model are also reported, showing that the properties of $Ca^{2+}$ transients and slow waves are particularly sensitive to the properties of $Ca^{2+}$ currents included in the model. Finally, the implications of these results are discussed in Section 6.3.

6.1 Model Development

6.1.1 Overview of the Baseline Cell Model

The baseline cell model presented in this study was chosen to represent the pacemaker activity of ICC-MY from murine small intestine, because mice are a common animal model for studying ICC electrophysiology. Slow waves in the murine small intestine typically occur at $16–30 \text{ min}^{-1}$ (Goto et al., 2004; Kito and Suzuki, 2003), but frequencies of $10–50 \text{ min}^{-1}$ have been observed (Thomsen et al., 1998). The slow waves recorded from ICC have a characteristic morphology: a rapid upstroke phase, in which the cell depolarises from a resting membrane potential between $−80$ and $−50 \text{ mV}$ to a peak potential between $−25$ and $0 \text{ mV}$ (Lees-Green et al., 2011b; Sanders et al., 2006); a plateau phase, in which the membrane potential is maintained close to the peak potential; and a repolarisation phase, in which the cell returns to the resting membrane potential. The overall morphology of a slow wave is determined by the summation of the individual ion currents active during the slow wave.

The baseline model is predicated on the fundamental hypothesis that Ano1 channels activated by SOCE initiate slow waves in ICC-MY. A model of this pacemaker hypothesis was developed, and was adapted to investigate the effects of varying the ion currents and $E_{Cl}$ in the model. Several key assumptions were made in the development of the baseline model, and the experimental evidence supporting each assumption is outlined.
below, followed by a summary of the pacemaker hypothesis.

The first assumption is that cyclical oscillations of Ca\(^{2+}\) in the ER and cytosol are an essential component of the ICC pacemaker mechanism. The importance of intracellular Ca\(^{2+}\) dynamics in ICC pacemaker activity, particularly Ca\(^{2+}\) release from and uptake into IP\(_3\)-mediated stores in the ER, is well established, as described in Section 2.4. Pacemaker activity is inhibited when Ca\(^{2+}\) uptake via the sarco-endoplasmic reticulum ATPase (SERCA) is blocked (Malysz et al., 2001; Ward et al., 2000). IP\(_3\)Rs and ongoing IP\(_3\) generation are essential for pacemaker activity in ICC, because slow waves are absent in gastric smooth muscle tissue from mice lacking type 1 IP\(_3\)R (Suzuki et al., 2000), and pacemaker activity is inhibited when phospholipase C blockers are used to inhibit IP\(_3\) production (Lowie et al., 2011; Malysz et al., 2001). Mitochondrial Ca\(^{2+}\) handling has also been proposed to play a role in slow wave generation (Ward et al., 2000), but more recent evidence from ICC in situ indicates that mitochondria may not be essential for Ca\(^{2+}\) cycling (Lowie et al., 2011), so they were not included in the model.

The second assumption is that store-operated Ca\(^{2+}\) entry is a key part of ICC pacemaker activity, as introduced in Section 2.5. The occurrence of SOCE following Ca\(^{2+}\) store depletion was demonstrated in ICC by inhibiting SERCA pumps with thapsigargin, which caused an increase in [Ca\(^{2+}\)], that was dependent on external Ca\(^{2+}\) influx (Torihashi et al., 2002). Furthermore, inhibition of Ca\(^{2+}\) oscillations by Ca\(^{2+}\)-free external solution and the SOC channel inhibitors SK&F 96365 and 2-APB also demonstrated the importance of SOCE (Liu et al., 2005a; Torihashi et al., 2002), although it should be noted that 2-APB has complex effects on Ca\(^{2+}\) influx and release. 2-APB has varying effects on Orai, but in general >10\(\mu\)M 2-APB inhibits CRAC channels, while low micromolar concentrations of 2-APB potentiate CRAC channels (Prakriya and Lewis, 2001; Smyth et al., 2010). At similar concentrations, 2-APB inhibits a variety of TRPC and TRPM channels (Kim et al., 2005; Xu et al., 2005). 2-APB inhibits IP\(_3\)Rs with an IC\(_{50}\) of 42\(\mu\)M–1 mM, depending on IP\(_3\) concentration (Bootman et al., 2002; Maruyama et al., 1997). The effects of 2-APB on ICC must be interpreted with caution, but 2-APB appears to be a more consistent and potent inhibitor of SOC channels than IP\(_3\)Rs, suggesting a role for SOCE in ICC pacemaking.
The third assumption is that Ano1 Ca\(^{2+}\)-activated Cl\(^-\) currents are crucial for the initiation of slow waves, evidenced by the lack of slow waves in Ano1 knockout mice (Hwang et al., 2009).

The fourth assumption is that Ano1 channels in ICC are colocalised with SOC channels, such that Ca\(^{2+}\) release from the ER causes SOCE, resulting in Ca\(^{2+}\)-dependent activation of nearby Ano1 channels. Ano1 gating occurs in a complex Ca\(^{2+}\) and voltage-dependent manner. The literature is not consistent, but in general the EC\(_{50}\) for Ca\(^{2+}\) decreases from 5.9 ± 2.5 µM at −100 mV to 0.4 ± 0.1µM at +100 mV (Xiao et al., 2011). To our knowledge [Ca\(^{2+}\)]\(_i\) has not been quantitatively measured in ICC, but global Ca\(^{2+}\) transients in ICC are likely to be similar in magnitude to those seen in other cell types, such as cardiac myocytes, in which [Ca\(^{2+}\)]\(_i\) typically increases from a resting level around 100 nM to a peak around 1 µM (Shaw and Colecraft, 2013). At this level of Ca\(^{2+}\), the Ano1 channel variant modelled in Chapter 5 is less than 10% activated at RMP (see Figure 5.1). Ano1 channels can be exposed to sufficiently high Ca\(^{2+}\) concentrations by being in close proximity to a Ca\(^{2+}\) source. For example, Ca\(^{2+}\)-activated Cl\(^-\) channels are activated by SOC channels in vascular SMC (Angermann et al., 2012) and Xenopus oocytes (Kuruma and Hartzell, 1999, 2000), and Ano1 channels were recently shown to be coupled to IP\(_3\)Rs in nociceptive sensory neurons (Jin et al., 2013). For this model, Ano1 channels are assumed to be colocalised with SOC channels in ICC because of the evidence detailed above about the importance of SOCE.

The final assumption is that T-type Ca\(^{2+}\) channels contribute to the upstroke phase of slow waves in ICC. A number of voltage-dependent ion channels have been identified in ICC-MY, and the role of these ion channels remains a matter of ongoing discussion (Beyder and Farrugia, 2012; Lees-Green et al., 2011b), but experimental evidence shows that T-type Ca\(^{2+}\) currents are important for the upstroke phase (Gibbons et al., 2009; Kito and Suzuki, 2003). In addition, Ca\(^{2+}\) influx through T-type Ca\(^{2+}\) channels can modulate the intrinsic Ca\(^{2+}\) cycle by activating quiescent IP\(_3\)Rs, enabling voltage-dependent coordination and propagation of slow waves (Sanders et al., 2006).

In summary, the proposed pacemaker cycle in this model begins when IP\(_3\)-mediated Ca\(^{2+}\) release from the ER leads to depletion of the ER Ca\(^{2+}\) stores, activating store-
operated Ca\(^{2+}\) entry via SOC channels. Ano1 channels colocalised with SOC channels are activated by the local rise in Ca\(^{2+}\) in microdomains surrounding the open SOC channels, causing depolarisation of the ICC. The depolarisation initiates a slow wave by activating voltage-dependent ion channels. The morphology of the slow wave, including the plateau phase and repolarisation, is determined by the balance of voltage- and Ca\(^{2+}\)-dependent ion currents. The pacemaker cycle concludes when Ca\(^{2+}\) influx via SOC channels and uptake via SERCA pumps is sufficient to refill the ER stores, resulting in deactivation of the SOC channels and Ano1 channels.

This pacemaker hypothesis shares similarities with previous pacemaker hypotheses (Sanders et al., 2006; Lees-Green et al., 2011b), notably the importance of ER Ca\(^{2+}\) handling, Ca\(^{2+}\) microdomains and the role of T-type Ca\(^{2+}\) channels.

The key components of this pacemaker hypothesis are incorporated in the baseline cell model, as illustrated in the schematic diagram of an ICC in Figure 6.1. Each component is described in more detail below. The model supports the pacemaker hypothesis if it can quantitatively and qualitatively reproduce experimentally observed results. If the model cannot adequately simulate slow waves, then the pacemaker hypothesis needs to be revised accordingly.

The baseline ICC model is a compartmental model in which the membrane potential within the cell and the ion concentrations within each subcellular compartment are homogeneous. The model was implemented using a Hodgkin-Huxley type formulation, in which the cell membrane lipid bilayer is represented as a capacitance \(C_m\), and the ion channels in the membrane are represented as conductances. The change in the transmembrane potential \(V_m\) over time depends on the total ionic current:

\[
\frac{dV_m}{dt} = -\frac{I_{\text{ion}}}{C_m},
\]

where \(I_{\text{ion}}\) is the sum of the individual ion currents through each class of ion channel in the cell. \(C_m\) was set to 25 pF, which is a representative value for a murine small intestinal ICC (Goto et al., 2004; Kim et al., 2002).

Each class of ion channel is represented as a variable conductor, where the conductance
Figure 6.1: Schematic diagram of the cell, including ion channels and transporters in the plasma membrane and endoplasmic reticulum (ER). The cell is divided into two compartments: the bulk cytosol and the ER. Ca\textsuperscript{2+} changes in the bulk cytosol are determined by the Ca\textsuperscript{2+} flux through the IP\textsubscript{3} receptors (IP\textsubscript{3}R) and sarco-endoplasmic reticulum ATPase (SERCA) pumps in the ER, and the T-type and store-operated Ca\textsuperscript{2+} (SOC) channels and plasma membrane Ca\textsuperscript{2+} ATPase (PMCA) in the cell membrane. The Ca\textsuperscript{2+} microdomain around the colocalised Ano1 and SOC channels is indicated by a dotted line. The microdomain is not modelled as a separate compartment but rather is directly dependent on the Ca\textsuperscript{2+} flux through the SOC channel. The membrane potential across the plasma membrane is governed by ion currents through the Ano1, SOC and T-type Ca\textsuperscript{2+} channels along with additional inward and outward currents passed by other channels as specified later.
6.1. Model Development

depends on the channel open probability as a function of voltage or \([\text{Ca}^{2+}]_i\). The whole cell current through each class of ion channel is proportional to the difference between \(V_m\) and the reversal potential of the channel. For an ion channel selective for a single ion, the reversal potential is equal to the Nernst or equilibrium potential of that ion. The equations for the open probability of each channel are given in Appendix B. The model equations were solved in MATLAB (R2012a).

6.1.2 Calcium Model

Two intracellular \(\text{Ca}^{2+}\) compartments were included in the model: bulk cytosol \(\text{Ca}^{2+}\) \([(\text{Ca}^{2+})_i]\) and an endoplasmic reticulum \(\text{Ca}^{2+}\) store \([(\text{Ca}^{2+})_{\text{ER}}]\), as shown in Figure 6.1. To model \(\text{Ca}^{2+}\) flux into and out of the ER, a simple model of intracellular \(\text{Ca}^{2+}\) dynamics was adapted from the oscillatory \(\text{Ca}^{2+}\) model developed by Wang et al. (2010a) for airway SMC. The specific components used were a sigmoidal model of the SERCA pump, and an \(\text{IP}_3\)R model based on the De Young and Keizer (1992) model. The ryanodine receptor was excluded because it appears to be less important for ICC pacemaker activity (Malysz et al., 2001). The equations describing the \(\text{IP}_3\)R and SERCA \(\text{Ca}^{2+}\) flux were unchanged from the model presented by Wang et al. (2010a), with parameter values modified to adjust the frequency of \(\text{Ca}^{2+}\) oscillations to an appropriate value for murine small intestine (see Appendix B for details).

6.1.3 Store-Operated \(\text{Ca}^{2+}\) Channel Model

The type of store-operated \(\text{Ca}^{2+}\) (SOC) channel that might be present in ICC is unknown. Activation of SOC channels occurs when STIM1 senses depletion of the ER \(\text{Ca}^{2+}\) store. The \([\text{Ca}^{2+}]_{\text{ER}}\) at which STIM1 activates is around 200–250\(\mu\)M (Brandman et al., 2007; Stathopulos et al., 2008). Measured Hill coefficients for activation of the STIM1-Orai1 complex by ER depletion range from 4 to 8 (Brandman et al., 2007; Luik et al., 2008). Therefore, a Hill function with a coefficient of 8 and \(K_d\) of 200\(\mu\)M was used to model SOC channel open probability as a function of \([\text{Ca}^{2+}]_{\text{ER}}\).

When STIM1 is activated it can oligomerise with either Orai or TRPC proteins to
6. ICC MODEL

form Ca\(^{2+}\) release activated Ca\(^{2+}\) (CRAC) channels or non-selective cation channels, respectively (Mignen et al., 2008; Ng et al., 2009). CRAC channels are highly selective for Ca\(^{2+}\) and native CRAC currents are very small (Smyth et al., 2010). In this model the Ca\(^{2+}\) influx via the SOC channel was important for activating Ano1, but the SOC channel itself was expected to make a negligible contribution to depolarising the membrane potential, as evidenced by the lack of slow waves in smooth muscle tissue from Ano1 knockout mice (Hwang et al., 2009). Therefore, the SOC channel was implemented as a highly Ca\(^{2+}\)-selective CRAC channel with a small conductance of 0.1 nS. The mechanism by which Ca\(^{2+}\) flux through the SOC channel acts on Ano1 is described below.

6.1.4 Anoctamin1 Ca\(^{2+}\)-Activated Cl\(^{-}\) Channel Model

The model of Ano1 Ca\(^{2+}\)-activated Cl\(^{-}\) currents described in Chapter 5 was used in the ICC model. The equations and parameters for the Ano1 model are detailed in Appendix B.3.

6.1.5 Calcium Microdomain Calculation

Ano1 channels are likely to be located within a Ca\(^{2+}\) microdomain near a Ca\(^{2+}\) channel. In previous models of ICC, microdomains were represented using a small compartment with separate differential equations for the microdomain Ca\(^{2+}\) concentration and the bulk cytosol [Ca\(^{2+}\)]\(_i\) (Corrias and Buist, 2008; Faville et al., 2009), or using a spatial representation of Ca\(^{2+}\) concentration throughout the microdomain and bulk cytosol (Means and Sneyd, 2010).

The compartmental method does not have a heavy computational load, although computational efficiency decreases as the number of microdomains represented increases (Corrias and Buist, 2008; Faville et al., 2009). However, the Ca\(^{2+}\) concentration calculated within the microdomain compartment is unlikely to be realistic. On the other hand, the spatial method provides greater accuracy in estimating Ca\(^{2+}\) concentration, but is computationally expensive (Means and Sneyd, 2010).

In the present study, the [Ca\(^{2+}\)]\(_i\) seen by the Ano1 channel due to SOC channel Ca\(^{2+}\) influx is represented using the analytical formulation for the steady-state solution to the
rapid buffering approximation of $[Ca^{2+}]_i$ near the entrance of a point source (Smith, 1996). This enables colocalised channels to be modelled without the additional computational expense or uncertainties introduced by spatial or compartmental methods.

The $[Ca^{2+}]$ seen by Ano1 ($Ca_{Ano1}$) was calculated using the solution for the $[Ca^{2+}]$ near a channel mouth with one mobile buffer in the cytosol (Smith, 1996). The solution depends on the bulk $Ca^{2+}$ concentration far away from the point source, i.e. $[Ca^{2+}]_i$ ($Ca_i$); the $[Ca^{2+}]$ at the mouth of the SOC channel ($Ca_{SOC}$); and the distance from the SOC channel to the Ano1 channel, which was set to 50 nm to provide sufficiently high $Ca_{Ano1}$. To ensure $Ca_{Ano1}$ represented the $[Ca^{2+}]$ seen by an individual Ano1 channel, $Ca_{SOC}$ was calculated by dividing the total flux of $Ca^{2+}$ into the cell through SOC channels by an estimate of the number of SOC channels or channel clusters in the cell, which was estimated to be 50 channel clusters, based on the estimation that there are approximately 50 separate $Ca^{2+}$ microdomains in an ICC (Faville et al., 2009).

6.1.6 Voltage-Gated Calcium Channel Model

The final component implemented in the baseline model was a voltage-dependent T-type $Ca^{2+}$ channel. This channel was modelled using the VDDR $Ca^{2+}$ channel model from the Corrias and Buist (2008) ICC model, except where otherwise stated.

6.1.7 Total Calcium Fluxes in the Cytosol

Cytosolic $Ca^{2+}$ in the model was determined by the $Ca^{2+}$ fluxes across the ER and plasma membranes, and cytosolic $Ca^{2+}$ buffers were assumed to be fast and linear. Thus, the total change in $[Ca^{2+}]_i$ for each of the model variations presented below, except where otherwise stated, was given by

$$\frac{dCa_i}{dt} = b_c (J_{IPR} - J_{SERCA} + J_{SOC} + J_{CaT} - J_{PMCA})$$  \hspace{1cm} (6.2)

where $b_c$ is the buffering constant in the cytosol, $J_{IPR}$ is the flux out of the ER through the IP$_3$R, $J_{SERCA}$ is the uptake into the ER, $J_{SOC}$ is the total flux through the SOC channels,
6. ICC MODEL

$J_{CaT}$ is the total flux through the voltage-gated T-type Ca$^{2+}$ channels, and $J_{PMCA}$ is the rate of extrusion of Ca$^{2+}$ out of the cell via the PMCA pump.

### 6.1.8 Model Variations

The baseline cell model was used to develop six model variations in order to investigate the currents active during the plateau phase and the effects of changing $E_{Cl}$.

$E_{Cl}$ in ICC-MY is reported to be around $-40$ to $-50$ mV (Zhu et al., 2010). However, previous estimates of $E_{Cl}$ were often higher than this (Tokutomi et al., 1995) and depolarising Ca$^{2+}$-activated Cl$^-$ currents were suggested to produce the plateau phase (Kito and Suzuki, 2003). In contrast, Cl$^-$ loading during microelectrode recordings may raise $[Cl^-]_i$ and $E_{Cl}$ above endogenous levels, as discussed in Section 6.3.2 (Blatt and Slayman, 1983). Therefore, two different levels of $E_{Cl}$ were tested: one in which $E_{Cl}$ is close to the plateau potential; and one in which $E_{Cl}$ is at the reported value of $-50$ mV (Zhu et al., 2010).

Ano1 Cl$^-$ currents can initiate the slow wave, and T-type Ca$^{2+}$ channels contribute to the upstroke, but the identity of the ion channels active during the plateau phase remain undetermined, so four hypothetical plateau currents were considered. These currents encompass a range of ion channel properties that could theoretically maintain a depolarising current at the peak potential of a slow wave, including currents that have thus far not been identified in ICC. The four scenarios considered were: (i) plateau current is carried by voltage-dependent Na$^+$ channels; (ii) plateau current is carried by voltage-dependent non-selective channels; (iii) plateau current is carried by Ca$^{2+}$-dependent non-selective channels that are activated by a voltage-dependent Ca$^{2+}$ influx; or (iv) plateau current is carried by voltage-dependent Ca$^{2+}$ channels.

The voltage-dependent Na$^+$ channel found in ICC, NaV1.5, inactivates rapidly upon depolarisation, so it is unlikely to contribute meaningfully to the plateau phase (Strege et al., 2003b). No long-lasting voltage-activated Na$^+$ channels have been identified in ICC to date, but this type of channel is theoretically capable of generating the plateau phase and is therefore included as a possible plateau current. A candidate Ca$^{2+}$ channel that may stay open long enough to generate the plateau current is the L-type Ca$^{2+}$ channel.
(Kim et al., 2002). The voltage- and Ca\(^{2+}\)-dependent non-selective channels may be either non-selective cation channels or maxi channels, both of which pass currents that reverse close to 0 mV. Non-selective cation channels in ICC are likely to be Ca\(^{2+}\)-dependent (Takeda et al., 2008). Maxi channels observed in ICC in situ are permeable to both Cl\(^-\) and Na\(^+\) ions, and display voltage-dependent activation and inactivation (Parsons et al., 2012).

Originally, a Cl\(^-\) current generated by the Ano1 channel was also considered as a fifth plateau current candidate. However, initial simulations found that a Cl\(^-\) current could not maintain the plateau in this ICC model unless the conductance was unrealistically large and E\(_{\text{Cl}}\) was unphysiologically positive, so this possibility was not considered plausible.

The six model variations have different hypotheses regarding the ion currents involved in generating slow waves, such that the “additional currents” mentioned in Figure 6.1 differ in each variation. The model variations are grouped into two categories based on [Cl\(^-\)]\(_i\) and the resultant E\(_{\text{Cl}}\):

**High-Cl:** E\(_{\text{Cl}}\) is close to the peak of the slow wave at \(-20\) mV. Ano1 current is responsible for initiating the slow wave, but an additional plateau current is included to maintain depolarisation through the peak and plateau. There were four variations to the High-Cl hypothesis:

1. A voltage-dependent Na\(^+\) current (I\(_{\text{NaV}}\)) acts to maintain depolarisation through the peak and plateau.

2. A voltage-dependent non-selective cation or maxi current (I\(_{\text{NSV}}\)) with a reversal potential of 0 mV maintains depolarisation through the peak and plateau.

3. The voltage-dependent T-type Ca\(^{2+}\) current, activated by the initial depolarisation caused by Ano1, contributes to the upstroke and produces a larger increase in [Ca\(^{2+}\)]\(_i\) than that due to Ca\(^{2+}\) influx through SOC channels alone. The Ca\(^{2+}\) transient activates a Ca\(^{2+}\)-dependent non-selective cation or maxi current (I\(_{\text{NSCa}}\)) with a reversal potential of 0 mV to maintain depolarisation through the peak and plateau.
4. A long-lasting voltage-dependent Ca\(^{2+}\) current (I\(_{CaV}\)) acts to maintain depolarisation through the peak and plateau.

**Low-Cl:** \(E_{Cl}\) is \(-50\) mV, as measured in ICC *in situ* (Zhu et al., 2010). Depolarisation to the peak potential and during the plateau phase is due to either a voltage-gated current or a Ca\(^{2+}\)-activated current. Based on the High-Cl simulation results, there was little value in testing I\(_{CaV}\) at the lower \(E_{Cl}\). The similarities between the High-Cl results with I\(_{NaV}\) and I\(_{NSV}\) and the effect of \(E_{Cl}\) on the simulation results, as discussed later, meant that a single voltage-gated current could be tested in the Low-Cl hypotheses without affecting the conclusions of the study. Therefore, the candidate plateau currents tested were:

1. A voltage-activated Na\(^{+}\) current, as in High-Cl(NaV), or
2. A Ca\(^{2+}\)-activated non-selective current that is activated by transient voltage-gated Ca\(^{2+}\) current, as in High-Cl(NSCa).

Repolarisation at the end of the plateau phase may be initiated by a combination of a delayed activation or increased amplitude of a K\(^{+}\) current or Ano1 current, and a voltage-, Ca\(^{2+}\)- or time-dependent inactivation of the inward plateau current.

The full complement of ion currents included in each model variation is detailed in the Section 6.2. The equations and parameters for each of these ion channels are given in Appendix B.

### 6.2 Results

#### 6.2.1 Impact of Altering \(E_{Cl}\)

The first step was to test the impact of varying \(E_{Cl}\) and the plateau current on the ICC model. Two values of \(E_{Cl}\) were tested: \(-20\) mV and \(-50\) mV, referred to as High-Cl and Low-Cl model variations, respectively. The results of each model variation are presented below.
Simulations were carried out using both the normal ("wild-type") cell model and an "Ano1 knockout" scenario in which the maximal whole cell conductance of Ano1 ($g_{Ano1}$) is set to 0 nS, in order to verify that Ano1 channels are essential for initiating the slow wave in each model variation.

The resting membrane potential, amplitude, frequency, and half-width (duration at half-maximal amplitude) were quantified, as summarised in Table 6.1. All the simulated slow waves have similar resting membrane potentials and amplitudes, and these values are within the expected range for ICC. The greatest variations in the quantified model results are in the frequency and half-width values, indicating that these temporal characteristics of the slow wave are more dependent than the membrane potential on the changes to $E_{Cl}$ and the ion currents in the model.

**High-Cl Simulations**

The four High-Cl model variations set $E_{Cl}$ at $-20$ mV, close to the peak potential of a slow wave. In addition to the three ion channels described in the baseline model—SOC channels ($I_{SOC}$), Ano1 channels ($I_{Ano1}$), and T-type Ca$^{2+}$ channels ($I_{CaT}$)—each High-Cl model variation incorporates a voltage-activated inward current to enable the plateau phase to be generated, as detailed below. Two "background" or "leak" channels are also included, a background K$^+$ current ($I_{Kb}$) and a background Na$^+$ current ($I_{Nab}$). $I_{Kb}$ acts to maintain the resting membrane potential and to repolarise the cell following a slow wave, while $I_{Nab}$ also contributes to maintenance of the resting membrane potential.

Each of the High-Cl model variations were tested in both the wild-type scenario, with normal Ano1 current, and in the Ano1 knockout scenario, in which Ano1 conductance is set to zero (Figure 6.2, black and grey lines, respectively). Slow wave-like potentials are successfully produced by the High-Cl(NaV) model variation, which incorporates a voltage-activated Na$^+$ channel (Figure 6.2A). Qualitatively similar results are obtained using a voltage-activated non-selective channel (High-Cl(NSV), see Figure 6.2C) or a Ca$^{2+}$-activated non-selective channel in conjunction with a transient voltage-activated Ca$^{2+}$ channel (High-Cl(NSCa), see Figure 6.2E), but not with a longer-lasting voltage-activated Ca$^{2+}$ channel (High-Cl(CaV), see Figure 6.2G), as described below.
Table 6.1: Quantitative characteristics of slow waves simulated by the six model variations. RMP is the resting membrane potential; Amplitude is the difference between resting membrane potential and peak slow wave potential; Half-width is the duration of slow wave as measured at half of the amplitude; Wild-type denotes the simulations using normal model variations; Knockout denotes Ano1 knockout simulations.

<table>
<thead>
<tr>
<th>Variation</th>
<th>RMP (mV)</th>
<th>Amplitude (mV)</th>
<th>Half-width (s)</th>
<th>Frequency (min(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild-type</td>
<td>Knockout</td>
<td>Wild-type</td>
<td>Wild-type</td>
</tr>
<tr>
<td>High-Cl</td>
<td>NaV</td>
<td>−66.4</td>
<td>−67.2</td>
<td>44.4</td>
</tr>
<tr>
<td></td>
<td>NSV</td>
<td>−64.6</td>
<td>−65.1</td>
<td>44.4</td>
</tr>
<tr>
<td></td>
<td>NSCa</td>
<td>−65.3</td>
<td>−65.1</td>
<td>41.9</td>
</tr>
<tr>
<td></td>
<td>CaV</td>
<td>−65.1</td>
<td>−63.2</td>
<td>48.8</td>
</tr>
<tr>
<td>Low-Cl</td>
<td>NaV</td>
<td>−66.7</td>
<td>−66.0</td>
<td>42.9</td>
</tr>
<tr>
<td></td>
<td>NSCa</td>
<td>−67.2</td>
<td>−65.0</td>
<td>47.6</td>
</tr>
</tbody>
</table>
Figure 6.2: Membrane potential and Ca$^{2+}$ oscillations produced from High-Cl model simulations with $E_{\text{Cl}}$ set to $-20$ mV. The plateau current is generated by a voltage-activated Na$^+$ channel (A–B), a voltage-activated non-selective channel (C–D), a Ca$^{2+}$-activated non-selective channel (E–F), or a voltage-activated Ca$^{2+}$ channel (G–H). Column 1: Membrane potentials simulated using the wild-type (WT) and Ano1 knockout (KO) scenarios (black and grey lines, respectively). Column 2: Oscillations in [Ca$^{2+}$]$_i$ simulated using the wild-type (WT) and Ano1 knockout (KO) scenarios (black and grey lines, respectively).
Using the High-Cl(NSV) model variation as an example, Figure 6.3 shows the contribution of the different ion currents to different phases of the simulated slow wave. The resting membrane potential is set by the balance of $I_{\text{Kb}}$ and $I_{\text{Na}}$. The upstroke phase is initiated by the pacemaker current, $I_{\text{Ano1}}$. As the membrane potential approaches $E_{\text{Cl}}$, $I_{\text{Ano1}}$ decreases to zero but the simultaneous increase in $I_{\text{NSV}}$ causes the upstroke depolarisation to continue. $I_{\text{NSV}}$, referred to as the plateau current, is the largest inward current active during the plateau phase. Repolarisation occurs as $I_{\text{NSV}}$ decreases in amplitude relative to $I_{\text{Kb}}$, although the increased driving force for $I_{\text{Ano1}}$ during repolarisation prolongs the time taken to return to resting membrane potential. $I_{\text{SOC}}$ and $I_{\text{CaT}}$ contribute little to the membrane potential, and their primary function is carrying Ca$^{2+}$ ions into the cell.

In the other model variations, the contribution of each current to different phases of the slow wave is predominantly the same as that shown in Figure 6.3, but with a different plateau current in place of $I_{\text{NSV}}$. There are two other notable differences. Firstly, in the Low-Cl model variations $I_{\text{Ano1}}$ contributes a large outward current during the plateau phase. Secondly, in the High-Cl(NSCa) and Low-Cl(NSCa) models, $I_{\text{CaT}}$ is large enough to make a meaningful contribution to depolarisation during the upstroke phase.

The background Na$^{+}$ current plays an important role in inhibiting membrane potential oscillations in the Ano1 knockout simulations. When $g_{\text{Ano1}}$ is set to zero in test simulations without the background Na$^{+}$ current, the depolarisations generated by the SOC current are tens of mV in amplitude (not shown), which runs counter to the key assumption that loss of Ano1 blocks slow waves.

As well as the Na$^{+}$ background channel, the efficacy of two other channels at inhibiting membrane potential oscillations in Ano1 knockout simulations were trialled. A voltage-activated K$^{+}$ channel with strong voltage-dependence at resting membrane potential and a Ca$^{2+}$-activated K$^{+}$ channel colocalised with the SOC channel both act to reduce membrane potential oscillations in the Ano1 knockout scenario, but these channels are less effective than the Na$^{+}$ leak channel (not shown).

**High-Cl(NaV): Sodium channel.** For the High-Cl(NaV) variation, a hypothetical voltage-activated, non-inactivating Na$^{+}$ current ($I_{\text{NaV}}$) is added to the model, so the total
Figure 6.3: Ion currents simulated using the High-Cl(NSV) model over the course of a single slow wave. The ion channels that contribute to membrane potential are store-operated Ca\textsuperscript{2+} channels (A), Ano1 Cl\textsuperscript{−} channels (B), T-type Ca\textsuperscript{2+} channels (C), background K\textsuperscript{+} channels (D), background Na\textsuperscript{+} channels (E), and a voltage-activated non-selective channel (F).
The ionic current across the membrane is

\[ I_{\text{ion}} = I_{\text{SOC}} + I_{\text{Ano1}} + I_{\text{CaT}} + I_{\text{Kb}} + I_{\text{Nab}} + I_{\text{NaV}}. \]  

(6.3)

The model produces slow wave-like depolarisations that peak 2.0 mV negative to \( E_{\text{Cl}} \) (Figure 6.2A; Table 6.1). Simulating Ano1 knockout results in a loss of slow waves, with residual oscillations smaller than 3 mV in amplitude (Figure 6.2A, grey line) and little change in the resting membrane potential or frequency (Table 6.1).

Cytosolic \( \text{Ca}^{2+} \) oscillations occur at the same frequency as slow waves and persist in Ano1 knockout simulations (Figure 6.2B). In the wild-type simulation, the bulk cytosolic \( \text{Ca}^{2+} \) concentration \( (\text{Ca}_i) \) peaks at 0.6 \( \mu \text{M} \), whereas the [\( \text{Ca}^{2+} \)] in the microdomain near the SOC channel mouth is approximately 14 times greater than this (not shown), enabling a much higher level of Ano1 activation than would occur if Ano1 channels were not localised near SOC channels.

**High-Cl(NSV): Voltage-activated non-selective channel.** Currents with reversal potentials close to 0 mV have been observed in ICC, either carried by non-selective cation channels (Goto et al., 2004; Koh et al., 2002) or maxi channels permeable to \( \text{Cl}^- \) and \( \text{Na}^+ \) (Parsons et al., 2012). Maxi channels in particular display activation and inactivation in response to voltage changes (Parsons et al., 2012). Thus, the High-Cl(NSV) model variation incorporates a voltage-dependent non-selective current \( (I_{\text{NSV}}) \) that reverses at 0 mV and displays voltage-dependent activation and partial inactivation. The total ionic current is

\[ I_{\text{ion}} = I_{\text{SOC}} + I_{\text{Ano1}} + I_{\text{CaT}} + I_{\text{Kb}} + I_{\text{Nab}} + I_{\text{NSV}}. \]  

(6.4)

The quantitative and qualitative characteristics of the membrane depolarisations generated by this model variation are comparable to those of slow waves generated by murine small intestine ICC. Membrane potential oscillations are reduced to 1.5 mV in amplitude in Ano1 knockout simulations, with little change in resting membrane potential or frequency (Figure 6.2C, grey line; Table 6.1). \( \text{Ca}^{2+} \) oscillations occur at the same frequency as the slow waves, and are maintained in the Ano1 knockout simulation (Figure 6.2D).

**High-Cl(NSCa): \( \text{Ca}^{2+} \)-activated non-selective channel.** \( \text{Ca}^{2+} \) dependence has
been identified in non-selective cation channels (Koh et al., 2002; Takeda et al., 2008). The Ca$^{2+}$ dependence of the maxi channel identified \textit{in situ} is unknown, although the maxi Cl$^{-}$ channel observed in cultured ICC is Ca$^{2+}$ dependent (Wright et al., 2012). The High-Cl(NSCa) model variation includes a Ca$^{2+}$-activated non-selective channel ($I_{\text{NSCa}}$) and the total ionic current is

\begin{equation}
I_{\text{ion}} = I_{\text{SOC}} + I_{\text{Ano1}} + I_{\text{CaT}} + I_{\text{Kb}} + I_{\text{Nab}} + I_{\text{NSCa}}.
\end{equation}

(6.5)

The parameters for $I_{\text{CaT}}$ in this model variation are modified in order to generate a larger voltage-dependent Ca$^{2+}$ influx, and the $K_d$ for the $I_{\text{NSCa}}$ current is set higher than the peak voltage-independent Ca$^{2+}$ amplitude. The resultant Ca$^{2+}$-activated non-selective channel is activated by voltage-dependent Ca$^{2+}$ influx in the wild-type scenario, generating slow waves that peak at $-23.4$ mV (Figure 6.2E, black line; Table 6.1). However, the channel remains relatively inactive during Ano1 knockout simulations in which the voltage-dependent Ca$^{2+}$ influx is negligible, so the amplitude of the residual membrane potential oscillations during Ano1 knockout simulations is reduced to $2.5$ mV (Figure 6.2E, grey line).

**High-Cl(CaV): Calcium channel.** The reversal potentials of both Na$^+$ and Ca$^{2+}$-selective currents are much more positive than the peak potential of a slow wave, suggesting that a long-lasting voltage-activated Ca$^{2+}$ current could be just as effective at generating the slow wave plateau phase as the voltage-activated Na$^+$ current used in High-Cl(NaV). The High-Cl(CaV) model variation incorporates a long-lasting voltage-gated Ca$^{2+}$ channel ($I_{\text{CaV}}$), so the total ionic current across the membrane is

\begin{equation}
I_{\text{ion}} = I_{\text{SOC}} + I_{\text{Ano1}} + I_{\text{CaT}} + I_{\text{Kb}} + I_{\text{Nab}} + I_{\text{CaV}}.
\end{equation}

(6.6)

Correspondingly, the change in cytosolic [Ca$^{2+}$]$_i$ is

\begin{equation}
\frac{d\text{Ca}_i}{dt} = b_c (J_{\text{IPR}} - J_{\text{SERCA}} + J_{\text{SOC}} + J_{\text{CaT}} + J_{\text{CaV}} - J_{\text{PMCA}}).
\end{equation}

(6.7)

The peak potential of depolarisations generated by this model variation is within the
expected range for an ICC-MY, and oscillations are abolished by Ano1 knockout (Figure 6.2G). However, the model exhibits a much faster initial repolarisation than that of a normal slow wave because of the impact of the long-lasting Ca$^{2+}$ influx on the intrinsic Ca$^{2+}$ dynamics (Figure 6.2H). Consequently, the results of the High-Cl(CaV) model variation differ from those of the other High-Cl models and will be considered in more detail in Section 6.2.2.

In summary, the High-Cl model variations generate membrane potentials with amplitudes comparable to slow waves from the mouse small intestine, and this activity is inhibited when simulating Ano1 knockout (Figure 6.2, column 1). Membrane potential oscillations occur at the same frequency as Ca$^{2+}$ oscillations (Figure 6.2, column 2). The Ano1 channel contributes negligible current at the slow wave peak in these model variations because the peak potential is close to $E_{Cl}$. The plateau phase is generated by a current with a more positive reversal potential, such as a non-selective or Na$^+$ current.

**Low-Cl Simulations**

In the High-Cl model variation $E_{Cl}$ is set at $-20\, \text{mV}$, but this is higher than the $E_{Cl}$ measured in ICC from the mouse small intestine (Zhu et al., 2010). Furthermore, none of the model variations presented so far are able to depolarise the cell past $E_{Cl}$ while retaining the characteristic plateau phase of the slow wave. The delayed repolarisation in the High-Cl model variations is facilitated by deactivation of the Ano1 current at the end of the Ca$^{2+}$ transient. The Low-Cl model variations set $E_{Cl}$ at $-50\, \text{mV}$, as measured in ICC-MY in situ (Zhu et al., 2010). The slow wave depolarises the ICC membrane potential past $E_{Cl}$, so a combination of time-, voltage- and Ca$^{2+}$-dependent currents is necessary to reproduce the plateau phase and initiate the repolarisation phase.

When developing the Low-Cl model variations, many different combinations of inward and outward currents were tested in an effort to reproduce ICC slow wave activity. The model variations described below represent two of the most successful attempts. Both produce cyclical depolarisations with an amplitude and frequency comparable to that of slow waves recorded from murine small intestine ICC-MY and similar to the simulated slow waves in the High-Cl model variations. However, the morphology of the depolarisations
produced by the Low-Cl model variations do not adequately match the expected slow wave morphology (Figure 6.4).

**Low-Cl(NaV): Sodium channel.** The Low-Cl(NaV) model variation uses a voltage-dependent Na$^+$ channel ($I_{NaV}$) to generate depolarisation during the plateau phase, similar to High-Cl(NaV). In addition, two voltage-gated K$^+$ currents are introduced, a transient K$^+$ current ($I_{Kt}$), and an ERG-like K$^+$ current ($I_{KERG}$) that activates upon repolarisation (Beyder and Farrugia, 2012). The total ion current is

$$I_{ion} = I_{SOC} + I_{Ano1} + I_{CaT} + I_{Kb} + I_{Nab} + I_{NaV} + I_{Kt} + I_{KERG}. \quad (6.8)$$

This model produces slow wave-like depolarisations with a similar resting membrane potential, amplitude and frequency to the previous model variations (Table 6.1). However, the “plateau” phase is steeper than expected for an ICC, and the slow wave duration at half-amplitude is 27–47% shorter than the High-Cl(NaV), High-Cl(NSV), and High-Cl(NSCa) model variations. In addition, repolarisation slows down when the cell repolarises past $-50 \text{ mV}$ as Ano1 current becomes inward again (Figure 6.4A).

In the Ano1 knockout simulations, the membrane potential oscillations are reduced to 3.9 mV in amplitude, with little change in the resting membrane potential and frequency, while Ca$^{2+}$ oscillations are retained (Figure 6.4A–B; Table 6.1).

**Low-Cl(NSCa): Ca$^{2+}$-activated non-selective channel.** The final model variation tested is a Low-Cl variation in which a Ca$^{2+}$-dependent non-selective channel is used to maintain depolarisation during the plateau in response to a larger voltage-dependent Ca$^{2+}$ influx, similar to High-Cl(NSCa).

Thus, the Low-Cl(NSCa) model variation incorporates a Ca$^{2+}$-activated non-selective current ($I_{NSCa}$), as well as a modified T-type Ca$^{2+}$ current to enable larger Ca$^{2+}$ influx to activate the non-selective current ($I_{CaT}$), and a Na$^+$/Ca$^{2+}$ exchanger to balance the larger Ca$^{2+}$ influx ($I_{NCX}$). The total ionic current is

$$I_{ion} = I_{SOC} + I_{Ano1} + I_{CaT} + I_{Kb} + I_{Nab} + I_{NSCa} + I_{NCX}. \quad (6.9)$$
Figure 6.4: Membrane depolarisations and Ca\(^{2+}\) oscillations produced from model simulations with $E_{\text{Cl}}$ set to $-50\text{ mV}$. The plateau current is carried by either a voltage-activated Na\(^{+}\) channel (A–B), or a Ca\(^{2+}\)-activated non-selective channel (C–D). Column 1: Membrane potentials simulated using the wild-type (WT) and Ano1 knockout (KO) scenarios (black and grey lines, respectively). Column 2: Oscillations in [Ca\(^{2+}\)]\(_i\) simulated using the wild-type (WT) and Ano1 knockout (KO) scenarios (black and grey lines, respectively).
The corresponding change in cytosolic $[\text{Ca}^{2+}]_i$ is

$$\frac{d\text{Ca}_i}{dt} = b_c (J_{\text{IPR}} - J_{\text{SERCA}} + J_{\text{SOC}} + J_{\text{CaT}} - J_{\text{PMCA}} - J_{\text{NCX}}). \quad (6.10)$$

The Low-Cl(NSCa) model variation generates depolarisations with a similar resting membrane potential, amplitude and frequency to the other model variations (Table 6.1). As with the Low-Cl(NaV) variation, the plateau phase is less pronounced than in the High-Cl models. The fast upstroke phase is also not captured well by the Low-Cl(NSCa) variation, and the slow wave duration at half-amplitude is 27–47% shorter than the High-Cl model durations (Figure 6.4C; Table 6.1).

During Ano1 knockout simulations the remaining membrane potential oscillations are 1.5 mV in amplitude and occur 43% faster than the wild-type oscillations, corresponding to the change in frequency in the $\text{Ca}^{2+}$ oscillations (Figure 6.4C–D; Table 6.1).

In summary, the amplitude and frequency of the wild-type depolarisations in the Low-Cl simulations are within the established range for murine small intestine slow waves, and Ano1 knockout simulations inhibit membrane depolarisation. However, the ability of the models to reproduce the characteristic plateau phase of a slow wave is less clear. Both the Low-Cl(NaV) and Low-Cl(NSCa) models rapidly repolarise immediately after the peak because repolarising currents are greater in magnitude than depolarising currents at the peak potential, resulting in a shorter slow wave duration than the High-Cl model variations. The Low-Cl(NSCa) model also does not adequately reproduce the fast upstroke phase of a slow wave. This reflects the altered balance of inward and outward currents compared to the High-Cl models as the Ano1 current reverses at $-50 \text{ mV}$.

### 6.2.2 Impact of Varying $\text{Ca}^{2+}$ Currents

In addition to testing the effect of altering $E_{\text{Cl}}$, this study also tests whether a variety of different ion currents are plausible candidates for the plateau current that maintains the plateau phase of the slow wave. When varying the plateau current, the impact of altering the $\text{Ca}^{2+}$ influx has a notable impact on the quantitative and qualitative characteristics of the simulated pacemaker activity in both wild-type and Ano1 knockout scenarios.
Three of the six model variations—High-Cl(NaV), High-Cl(NSV), and Low-Cl(NaV)—have a relatively small voltage-dependent Ca\textsuperscript{2+} influx carried by the T-type Ca\textsuperscript{2+} channel. The Ca\textsuperscript{2+} oscillations produced by these variations all have similar amplitudes and frequencies. In the wild-type simulations the magnitude of Ca\textsuperscript{2+} oscillations is smaller and the frequency is faster than in the Ano1 knockout simulations (Figures 6.2B, 6.2D, and 6.4B; Table 6.1).

The three other model variations—High-Cl(NSCa), High-Cl(CaV), and Low-Cl(NSCa)—have a comparatively larger voltage-dependent Ca\textsuperscript{2+} influx carried by either a modified version of the T-type Ca\textsuperscript{2+} channel (High-Cl(NSCa) and Low-Cl(NSCa)) or a longer-lasting Ca\textsuperscript{2+} current designated I\textsubscript{CaV} (High-Cl(CaV)). In these variations, larger and slower Ca\textsuperscript{2+} oscillations are generated in the wild-type simulations than in the Ano1 knockout scenario; for example, the High-Cl(NSCa) wild-type Ca\textsuperscript{2+} oscillations peak at 1.9\,\mu\text{M} compared with 0.53\,\mu\text{M} in Ano1 knockout simulations. The exception is the High-Cl(CaV) model variation, which does not oscillate in the Ano1 knockout scenario (Figures 6.2F, 6.2H, and 6.4D; Table 6.1).

Furthermore, the wild-type Ca\textsuperscript{2+} oscillations produced by the three models with large Ca\textsuperscript{2+} influx are larger in amplitude and slower in frequency than those from the four smaller Ca\textsuperscript{2+} influx models. For example, in wild-type simulations with High-Cl(NSCa), [Ca\textsuperscript{2+}]\text{\textsubscript{i}}, peaks at 1.9\,\mu\text{M} (Figure 6.2F), whereas with High-Cl(NaV) and High-Cl(NSV) [Ca\textsuperscript{2+}]\text{\textsubscript{i}}, peaks at 0.6\,\mu\text{M} (Figure 6.2B and D).

In the Low-Cl(NSCa) model variation, the Ca\textsuperscript{2+} oscillations in the wild-type simulations are similar in magnitude and frequency to those from the High-Cl(NSCa) variation, due to the similarities in the Ca\textsuperscript{2+}-activated non-selective plateau current in these two models. Conversely, the Ca\textsuperscript{2+} oscillations in the Low-Cl(NSCa) Ano1 knockout simulations are smaller and faster than in any of the other model variations because of the additional Ca\textsuperscript{2+} extrusion mechanism—the plasma membrane NCX—included in this model (Figure 6.4D).

The High-Cl(CaV) model variation has a significantly larger Ca\textsuperscript{2+} influx than the other variations, with wild-type [Ca\textsuperscript{2+}]\text{\textsubscript{i}}, peaking as high as 4.7\,\mu\text{M}, compared with 1.9\,\mu\text{M} in High-Cl(NSCa) or 0.6\,\mu\text{M} in High-Cl(NaV) and High-Cl(NSV) (Figure 6.2, column 2). As a result, the membrane potential and Ca\textsuperscript{2+} oscillations are significantly slower in
the High-Cl(CaV) model variation, and in the Ano1 knockout simulation the lack of voltage-dependent Ca\(^{2+}\) entry results in complete loss of Ca\(^{2+}\) oscillations. Furthermore, while the resting membrane potential and amplitude of the depolarisations are within an appropriate range for slow waves recorded from ICC, the frequency and the morphology of the depolarisations are not. In particular, the characteristic plateau phase is not evident and instead a rapid repolarisation follows immediately after the peak of the upstroke phase (Figure 6.2G–H; Table 6.1).

The main pattern that emerges from these results is that simulations with larger Ca\(^{2+}\) oscillations—corresponding to larger Ca\(^{2+}\) influx—tend to have slower frequencies; this holds true for both wild-type and Ano1 knockout simulations. Consequently, the model variations that have a larger voltage-dependent Ca\(^{2+}\) influx have more substantial changes in frequency in Ano1 knockout compared with wild-type simulations, associated with significant changes in the amplitude of Ca\(^{2+}\) oscillations in the absence of voltage-dependent Ca\(^{2+}\) influx (Figures 6.2–6.4; Table 6.1).

6.3 Discussion

To our knowledge, this is the first mathematical model of ICC to implement Ano1 as a pacemaker channel and to incorporate store-operated Ca\(^{2+}\) entry.

6.3.1 ICC Model Development

One of the general limitations of modelling biophysical processes is that models can be sensitive to minor parameter changes, and care must be taken to ensure this does not inadvertently alter the model results. In the process of developing this ICC model it became apparent that the most important parameter was the Cl\(^{-}\) reversal potential. Altering E\(_{\text{Cl}}\) has a significant impact on the ability of the model to generate slow waves in response to an initial depolarisation by Ano1 channels. Furthermore, the channels responsible for maintaining depolarisation during the plateau phase have not yet been identified. Consequently, instead of presenting a single model of ICC pacemaker activity, the dependence of the baseline model on E\(_{\text{Cl}}\) was explored in depth by developing six
different model variations.

Voltage-gated Ca\(^{2+}\) influx is also shown to be a significant factor in the ability to generate slow waves. However, the qualitative results of the High-Cl model variations are relatively insensitive to model parameters other than E\(_{\text{Cl}}\). As shown in Figure 6.2, it is possible to simulate slow wave-like depolarisations when the plateau current is carried by a voltage-gated Na\(^{+}\) channel, a voltage-gated non-selective channel, or a Ca\(^{2+}\)-activated non-selective channel. The exception is the High-Cl(CaV) model variation that includes a large voltage-gated Ca\(^{2+}\) current, which is unable to generate slow wave-like events because of the effect of the large Ca\(^{2+}\) influx on the intrinsic Ca\(^{2+}\) cycle.

Conversely, with the Low-Cl model variations the ability to reproduce slow wave-like events is highly dependent on the parameters used. Many of the parameter sets tested were unable to generate slow waves at all. However, those that were able to generate oscillatory depolarisations produced qualitatively and quantitatively similar results to those shown in Figure 6.4, in which the plateau phase is not well reproduced.

Therefore, although a single pacemaker hypothesis underlies each of the model variations, this model does not present an exact replication of the pacemaker mechanism in ICC. Rather, the model was used to assess the viability of some of the components and processes that may contribute to ICC slow wave activity. The ability of four candidate ion channels to generate the plateau phase of the slow wave in response to an initial depolarisation by Ano1 current was tested, and how E\(_{\text{Cl}}\) affects this mechanism was considered.

### 6.3.2 Impact of Altering E\(_{\text{Cl}}\)

The High-Cl(NaV), High-Cl(NSV), and High-Cl(NSCa) model variations present mathematically plausible mechanisms for generating slow waves, thereby supporting the hypothesis that Ano1 acts as a pacemaker channel.

Conversely, the quantitative characteristics of the Low-Cl models are within the appropriate range for slow waves (Table 6.1) but the results do not qualitatively resemble ICC slow waves (Figure 6.4A and C). In particular, the prolonged plateau phase of slow waves is not adequately reproduced by the Low-Cl models, because the large outward...
current carried by Ano1 at the peak potential contributes to rapid repolarisation.

The simulation results indicate that a typical slow wave morphology is more readily produced when $E_{Cl}$ is near or above the peak slow wave potential, whereas the ability to generate slow waves is complicated by lowering $E_{Cl}$ well below the peak potential. The short slow wave duration of the Low-Cl model variations restricts their use in other simulations, but the results can inform further experimental research.

Factors Affecting Cl\textsuperscript{−} Equilibrium Potential

Previous estimates of the equilibrium or reversal potentials of Cl\textsuperscript{−} currents in ICC varied widely, ranging from $-10$ mV (Tokutomi et al., 1995) to $-52$ mV (Park et al., 2005). Experimental (Kito and Suzuki, 2003) and computational (Corrias and Buist, 2008) studies have suggested that Cl\textsuperscript{−} channels contribute an inward current to maintain the plateau phase of the slow wave.

Subsequent measurements of $[\text{Cl}^-]_i$ in ICC-MY from murine jejunum show that $E_{Cl}$ is around $-50$ mV \textit{in situ} or $-41$ mV in explant culture (Zhu et al., 2010), suggesting that Cl\textsuperscript{−} channels generate outward current during the plateau phase and could contribute to repolarisation of the slow wave. However, the model results presented here suggest that the reversal potential of Ano1 Cl\textsuperscript{−} current is likely to be closer to the slow wave peak potential than to resting membrane potential. The finding that $E_{Cl}$ is $-41$ to $-50$ mV has yet to be replicated, but there are two hypothetical explanations for an apparent discrepancy between the experimentally measured $E_{Cl}$ and the reversal potential of Cl\textsuperscript{−} currents in this model.

Firstly, it is possible that the $[\text{Cl}^-]_i$ measured \textit{in situ} does not accurately reflect the $[\text{Cl}^-]_i$ seen by Ano1 channels during slow wave recordings. During microelectrode recordings, $[\text{Cl}^-]_i$ may be raised by the high KCl concentration within the microelectrode tip. Glass microelectrodes are typically filled with an aqueous solution of 3 M KCl in order to minimise electrode resistance—which helps to minimise the electrical time constant of the electrode—and to minimise tip potentials (Blatt and Slayman, 1983). This highly concentrated filling solution causes a leak of K\textsuperscript{+} and Cl\textsuperscript{−} ions into the impaled cell. The result is Cl\textsuperscript{−} loading, in which significant changes in $[\text{Cl}^-]_i$ and $E_{Cl}$ can occur, thus altering
the reversal potential of Cl\(^-\) currents (Kirby et al., 2000). For example, Cl\(^-\) leak rates of 4.4 fmol s\(^-1\) and elevations in [Cl\(^-\)]\(_i\) up to 72\(\mu\)M min\(^-1\) were observed in nonexcitable cells when using a 1 M KCl electrode filling solution (Blatt and Slayman, 1983). In a cell approximately 1 pL in volume—the estimated volume of a murine ICC (Corrias and Buist, 2008; Youm et al., 2006)—a leak rate on the order of 1 fmol s\(^-1\) will cause up to a 1 mM s\(^-1\) rise in [Cl\(^-\)]\(_i\), which corresponds to a 4–8% increase per second in ICC-MY where [Cl\(^-\)]\(_i\) is reported to be 12.8 to 26.1 mM (Zhu et al., 2010). Even accounting for mechanisms to balance [Cl\(^-\)]\(_i\), such as diffusion out of the cell or the action of Cl\(^-\) transporters, a 1 mM s\(^-1\) Cl\(^-\) leak into the cell can have a dramatic effect on the reversal potential of Cl\(^-\) currents.

Physiological [Cl\(^-\)]\(_i\) can also be altered in whole-cell patch clamp recordings of slow waves in cultured and isolated ICC, due to dialysis of the intracellular solution with the pipette solution (Liem et al., 1995). Manipulation of [Cl\(^-\)]\(_i\) using whole-cell patch clamp has been used in some studies to identify the Cl\(^-\) selectivity of ion currents (Zhu et al., 2009), but in other studies dialysis may alter the physiological [Cl\(^-\)]\(_i\), making it difficult to determine the precise behaviour of Cl\(^-\) currents and slow waves in ICC.

An important implication of \(E_{Cl}\) being raised by a Cl\(^-\) leak into the cell is that the slow wave morphology observed during microelectrode recordings may not accurately reflect the true morphology of slow waves in ICC. This could account for the difficulties encountered in reproducing the typical slow wave morphology when \(E_{Cl}\) was set to \(-50\) mV in the Low-Cl model variations. On the other hand, the arrangement of ICC networks in a syncytium allows for diffusion of Cl\(^-\) ions between cells, which would mitigate the extent to which Cl\(^-\) leak from a microelectrode impacts [Cl\(^-\)]\(_i\) in the impaled cell (Fromm and Schultz, 1981; Liu et al., 1993). Furthermore, the results of membrane potential recordings do not appear to be markedly affected by differences in experimental setup, such as electrode resistance, concentration of electrode filling solution, or the use of ruptured versus perforated patch-clamp (e.g. Dickens et al., 1999; Goto et al., 2004; Kito et al., 2002a).

Secondly, an alternative explanation for a discrepancy between the [Cl\(^-\)]\(_i\) measured \textit{in situ} (Zhu et al., 2010) and the [Cl\(^-\)]\(_i\) that facilitates simulation of slow wave-like
depolarisations in this study is that Ano1 channels are located within Cl\textsuperscript{−} microdomains. Microdomains of Ca\textsuperscript{2+} are most common because of the highly buffered state of Ca\textsuperscript{2+} in the cytosol, but microdomains of other ions also occur, particularly close to ion transporters, such as Na\textsuperscript{+} microdomains observed near Na\textsuperscript{+}/K\textsuperscript{+} ATPase and NCX (Hauck and Frishman, 2012). Cl\textsuperscript{−} microdomains in ICC could form due to accumulation of Cl\textsuperscript{−} by ion transport proteins in the cell membrane. ICC-MY are known to express several Cl\textsuperscript{−} transporters, including a Na-K-Cl cotransporter (NKCC1) and three varieties of K-Cl cotransporters (Chen et al., 2007; Wouters et al., 2006). NKCC1 can maintain [Cl\textsuperscript{−}]\textsubscript{i} above electrochemical equilibrium, and in murine small intestine ICC-MY this function is critical for the pacemaker mechanism (Wouters et al., 2006). Local variations in [Cl\textsuperscript{−}]\textsubscript{i} may also be mediated by cytoplasmic impermeant anions and polyanionic extracellular matrix glycoproteins, as recently shown in neurons (Glykys et al., 2014).

6.3.3 Impact of Varying Ca\textsuperscript{2+} Currents

Other than E\textsubscript{Cl}, the main determinant of the viability of the model variations presented in this study was the magnitude of Ca\textsuperscript{2+} influx associated with the plateau current.

The High-Cl(CaV) model variation, which includes a long-lasting voltage-activated Ca\textsuperscript{2+} current large enough to depolarise the cell above −20 mV, is not a valid model of slow wave activity. The large Ca\textsuperscript{2+} influx that accompanies depolarisation alters the intrinsic Ca\textsuperscript{2+} cycle, resulting in low frequency spiking events instead of slow waves (Figure 6.2G), and generating Ca\textsuperscript{2+} transients much larger than those observed in other cell types (Shaw and Colecraft, 2013), but a smaller Ca\textsuperscript{2+} current would not cause sufficient depolarisation for a slow wave. Thus, Ca\textsuperscript{2+} currents are unlikely to contribute a major component of the plateau current.

However, it is plausible that a voltage-gated Ca\textsuperscript{2+} current is responsible for activating the plateau current, as in the High-Cl(NSCa) and Low-Cl(NSCa) model variations (Figures 6.2E–F and 6.4C–D). The Ca\textsuperscript{2+} transients in these two model variations are somewhat larger than those observed in cardiac myocytes (Shaw and Colecraft, 2013). If the plateau channel model was more sensitive to Ca\textsuperscript{2+}, it could be activated by the
smaller voltage-dependent Ca\(^{2+}\) influx used in the High-Cl(NaV) and High-Cl(NSV) model variations, but this would obviate the need for Ano1 to initiate slow waves.

Alternatively, the Ca\(^{2+}\)-activated non-selective channel could be colocalised with the T-type Ca\(^{2+}\) channel. This is consistent with previous pacemaker hypotheses, in which the plateau phase occurs in response to voltage-dependent Ca\(^{2+}\) influx via T-type Ca\(^{2+}\) channels located in microdomains with IP\(_3\)Rs and Ca\(^{2+}\)-dependent cation channels (Lees-Green et al., 2011b; Sanders et al., 2006). Ca\(^{2+}\) influx through T-type Ca\(^{2+}\) channels during the upstroke phase is proposed to stimulate further IP\(_3\)R-mediated Ca\(^{2+}\) release from the ER to activate unitary potentials, which fired in a coordinated manner to form the plateau phase, as introduced in Section 2.7–2.8. This hypothesis was supported by experimental evidence that inhibiting Ca\(^{2+}\) handling inhibited the plateau phase (Hirst and Edwards, 2001; Lee et al., 2007b), and that voltage-dependent Ca\(^{2+}\) influx through T-type Ca\(^{2+}\) channels appeared to trigger regeneration of the intrinsic Ca\(^{2+}\) release mechanism from stores in the ER during propagation of Ca\(^{2+}\) waves in ICC networks (Lee et al., 2007b; Park et al., 2006; Sanders et al., 2006). The formation of the plateau phase by unitary potentials has been successfully modelled previously (Faville et al., 2009). Modelling the generation of unitary potentials with microdomains containing T-type Ca\(^{2+}\) channels and the plateau channel was outside the scope of the present study, but can be explored in future work.

Mathematically feasible models of pacemaker activity in which voltage-dependent Ca\(^{2+}\) influx does not play a crucial role are also presented here. In the High-Cl(NaV) model variation the plateau current is a voltage-dependent Na\(^{+}\) current, and in the High-Cl(NSV) model variation the plateau current is a voltage-dependent non-selective current (Figure 6.2A and C). T-type Ca\(^{2+}\) channels are present but are not essential for producing the plateau phase, or for the Ca\(^{2+}\) cycle, as evidenced by the continuation of Ca\(^{2+}\) oscillations in the Ano1 knockout simulations. Nevertheless, Ca\(^{2+}\) influx via SOC channels remains an intrinsic component of the pacemaker mechanism in these model variations.

Although T-type Ca\(^{2+}\) channels may not be crucial for generating slow waves in isolated ICC, voltage-dependent Ca\(^{2+}\) currents appear to play an important role in the
propagation of slow waves and Ca$^{2+}$ waves through ICC networks (Park et al., 2006; Sanders et al., 2006; Ward et al., 2004). This ICC model can be used to simulate the behaviour of ICC networks in future studies.

The Role of Voltage-Gated Ca$^{2+}$ Currents in ICC

Determining the precise role of voltage-gated Ca$^{2+}$ channels in ICC is complicated by conflicting reports in the effects of inhibiting Ca$^{2+}$ influx. Kito and Suzuki (2003) showed in mouse small intestine tissue segments that exposure to nominally Ca$^{2+}$-free external solution for over 20 minutes decreased slow wave amplitude, upstroke rate, and frequency but did not completely block slow waves. This suggests that a physiological concentration of extracellular Ca$^{2+}$ is not essential for slow wave generation, although caveolae may act as a source of external Ca$^{2+}$ in nominally Ca$^{2+}$-free solutions (Daniel et al., 2009).

On the other hand, Ward et al. (2004) showed in canine gastric antrum muscle strips that high concentrations of Ni$^{2+}$ and mibefradil, and prolonged exposure to Ca$^{2+}$-free solution abolished slow waves completely, indicating that external Ca$^{2+}$ influx is a crucial component of the pacemaker mechanism. The contribution of voltage-gated Ca$^{2+}$ channels to intracellular Ca$^{2+}$ transients in ICC also remains unclear. In mouse small intestine ICC-MY, one study demonstrated that inhibiting T-type Ca$^{2+}$ channels with 100µM Ni$^{2+}$ or 0.1 to 1µM mibefradil reduced the frequency, amplitude and propagation of Ca$^{2+}$ transients and eventually blocked all activity (Park et al., 2006), while in another study 0.5µM mibefradil was shown to have no effect on Ca$^{2+}$ transients (Lowie et al., 2011). On the other hand, L-type Ca$^{2+}$ channel blockers alone did not inhibit Ca$^{2+}$ transients in ICC-MY (Lee et al., 2007b), but when T-type Ca$^{2+}$ channels were inhibited with 0.05µM mibefradil, blocking L-type Ca$^{2+}$ channels with nicardipine further inhibited Ca$^{2+}$ transients (Park et al., 2006). Thus, L-type channels may help to maintain Ca$^{2+}$ transients when T-type Ca$^{2+}$ entry is inhibited (Park et al., 2006).

The pharmacological agents used in these studies cannot conclusively differentiate between the role of T-type Ca$^{2+}$ channels and other Ca$^{2+}$ channels. Low [Ca$^{2+}$]o impacts the function all Ca$^{2+}$ channels, while mibefradil can inhibit L-type Ca$^{2+}$ channels (Strege
et al., 2005) and Ni\(^{2+}\) can inhibit store-operated (Flemming et al., 2003) and receptor-mediated (Inoue, 1991) Ca\(^{2+}\) influx.

In the present study, inhibition of the baseline T-type Ca\(^{2+}\) channel during Ano1 knockout simulations has little effect on the Ca\(^{2+}\) transient amplitude in the High-Cl\((NaV)\), High-Cl\((NSV)\), and Low-Cl\((NaV)\) variations (Figures 6.2B, 6.2D, and 6.4B), in agreement with the experimental observation that T-type Ca\(^{2+}\) entry does not contribute significantly to Ca\(^{2+}\) transients in ICC (Lowie et al., 2011). In contrast, in the High-Cl\((NSCa)\) and Low-Cl\((NSCa)\) model variations, the T-type Ca\(^{2+}\) channel model is modified to increase the voltage-dependent Ca\(^{2+}\) influx, and the decreased amplitude of Ca\(^{2+}\) transients in the Ano1 knockout simulations (Figures 6.2F and 6.4D) corresponds with the experimental observation that Ca\(^{2+}\) transients are inhibited by inhibiting T-type Ca\(^{2+}\) channels (Lee et al., 2007b; Park et al., 2006).

**Effects of Ca\(^{2+}\) Influx on Ano1 Knockout Simulations**

All the model variations except for High-Cl\((CaV)\) demonstrate continuation of Ca\(^{2+}\) oscillations in the Ano1 knockout simulations. This suggests that Ca\(^{2+}\) oscillations can occur independently of slow waves, in agreement with the observation of rhythmic Ca\(^{2+}\)-dependent currents in ICC under whole cell voltage clamp (Thomsen et al., 1998; Tokutomi et al., 1995). The Ca\(^{2+}\) oscillations in Ano1 knockout simulations result from cyclical release and uptake of ER Ca\(^{2+}\) combined with SOCE. The frequency and magnitude of the Ca\(^{2+}\) oscillations in Ano1 knockout simulations also depends on the properties of the voltage-gated Ca\(^{2+}\) channel and the plasma membrane Ca\(^{2+}\) extrusion mechanism included in each model variation.

In the High-Cl\((NaV)\), High-Cl\((NSV)\), and Low-Cl\((NaV)\) model variations the amplitude of Ca\(^{2+}\) oscillations in the wild-type simulation is smaller than in the Ano1 knockout simulation (Figures 6.2B and D, and 6.4B). This result is unexpected, because the Ca\(^{2+}\) cycle in the wild-type simulations is augmented by a voltage-dependent Ca\(^{2+}\) influx that is inactive in the Ano1 knockout simulations. However, the increased amplitude of Ca\(^{2+}\) oscillations in the Ano1 knockout scenario is due to an increased driving force for SOCE at the more negative membrane potentials.
Conversely, in the High-Cl(NSCa) and Low-Cl(NSCa) model variations, the amplitude of Ca\(^{2+}\) oscillations in the wild-type simulations is much larger than in the Ano1 knockout simulations because of the larger voltage-dependent Ca\(^{2+}\) influx in the wild-type simulations (Figures 6.2F and 6.4D). These model variations also demonstrate increased frequencies in Ano1 knockout simulations resulting from the decreased amplitude of Ca\(^{2+}\) oscillations, likely due to the greater capacity of the plasma membrane Ca\(^{2+}\) pumps (see Table B.2 in Appendix B).

The 43\% increase in frequency in the Low-Cl(NSCa) Ano1 knockout simulations appears to contradict experimental evidence showing that Ca\(^{2+}\)-dependent currents recorded from ICC under voltage clamp oscillate at similar frequencies to slow waves (Thomsen et al., 1998; Tokutomi et al., 1995). However, the impact of Ca\(^{2+}\) influx on the simulated Ca\(^{2+}\) transient depends on the Ca\(^{2+}\) model used. The Low-Cl(NSCa) model variation includes simple phenomenological models of two Ca\(^{2+}\) extrusion mechanisms: a high-affinity PMCA and a low affinity NCX (Livshitz et al., 2012). Introducing a more complex model of an adaptive Ca\(^{2+}\) extrusion mechanism may enable the slow wave model to adjust to the changes in Ca\(^{2+}\) influx observed in the wild-type and Ano1 knockout simulations in order to maintain similar frequencies of Ca\(^{2+}\) oscillations.

Given that Ca\(^{2+}\) oscillations can occur without slow waves, the question of how these cell-wide intracellular Ca\(^{2+}\) transients are coordinated remains. Ca\(^{2+}\) waves travel around 20\(\mu\)m s\(^{-1}\) at 20\(^\circ\)C, and can travel more than four times faster at body temperature (37\(^\circ\)C) (Jaffe, 2010). In contrast, Ca\(^{2+}\) waves in ICC networks have been observed to propagate at 2 to 4 mm s\(^{-1}\) (Lee et al., 2007b; Park et al., 2006) and slow waves can propagate at 5 to 40 mm s\(^{-1}\) (Sanders et al., 2006). If a voltage signal is not required for coordinating intracellular Ca\(^{2+}\) dynamics, another rapid process must be involved. One possible mechanism for coordinating intracellular Ca\(^{2+}\) transients is via ER depletion. Modelling has shown that sharp gradients in localised \([\text{Ca}^{2+}]_{\text{ER}}\) are smeared out within milliseconds by buffering (Means et al., 2006), which means localised ER Ca\(^{2+}\) release and depletion could lead to ER depletion throughout the cell and coordination of the intracellular Ca\(^{2+}\) pacemaker signal.
6. ICC MODEL

6.3.4 Impact of Ano1 Knockout on Slow Waves

One of the key assumptions of the baseline model is that slow waves should be absent in an Ano1 knockout scenario. In recordings from Ano1 knockout mice the membrane potential of SMC is quiescent (Hwang et al., 2009). However, to our knowledge, there have been no membrane potential recordings from ICC in Ano1 knockout mice, so we cannot be certain that the absence of slow waves in SMC reflects a complete loss of slow waves in ICC.

In the Ano1 knockout simulations presented here, slow wave activity is inhibited but membrane potential oscillations are not completely abolished, except in the High-Cl(CaV) model in which both membrane potential and Ca\(^{2+}\) oscillations ceased. The residual membrane potential oscillations in the Ano1 knockout scenarios are generated primarily by the cyclical activation and deactivation of the SOC current in response to changes in ER Ca\(^{2+}\) (Figures 6.2 and 6.4). Therefore, the simulation results predict that Ano1 knockout mice retain small Ca\(^{2+}\)-driven depolarisations that are not transmitted to the SMC.

Leak currents are required to minimise the depolarising effect of SOC current during Ano1 knockout simulations. The Na\(^+\) leak current may be carried by a Na\(^+\) leak channel (NALCN) recently identified in murine small intestine ICC (Kim et al., 2012). The K\(^+\) leak current is likely to be carried by KCNK3 (also called TASK-1), which encodes a background K\(^+\) channel that is abundantly expressed in ICC-MY (Chen et al., 2007).

Alternative Roles for Ano1

The model assumes that the main function of Ano1 in ICC is to initiate slow waves by depolarising the cell from the resting membrane potential. However, Ano1 may contribute in other ways to the viability of ICC as pacemaker cells. Cl\(^-\) has been found to act as a second-messenger, particularly through the elevation of [Cl\(^-\)]\(_i\) via NKCC1, and appears to play a role in cell growth in a variety of cell types including fibroblasts, vascular and airway SMC, and lymphocytes (Orlov and Hamet, 2006). Ano1 in ICC has been shown to facilitate proliferation and this function is dependent on Cl\(^-\) entry (Stanich et al., 2011). Therefore, it is possible that the loss of slow waves in Ano1 knockout mice is due to an inhibitory effect on ICC proliferation rather than a simple loss of the pacemaker channel.
Nevertheless, Gao et al. (2013) quantitatively analysed wild-type and Ano1 knockout ICC networks and found that there were no significant differences in the topology of the network structures, so any impairment in the viability of Ano1 knockout ICC as pacemaker cells is likely to occur on the single-cell level.

The primary function of Ano1 currents in ICC is likely to be regulating voltage-gated ion channels by altering membrane potential. However, Cl$^{-}$ ions may also affect ER Ca$^{2+}$ handling by compensating for the charge build-up that occurs during Ca$^{2+}$ uptake (Pollock et al., 1998). Therefore, the influx of Cl$^{-}$ ions through Ano1 channels could influence the pacemaker cycle by directly modulating ER Ca$^{2+}$ uptake.

6.3.5 Role of Store-Operated Ca$^{2+}$ Entry

Another of the key assumptions made in the model development is that slow waves are initiated when Ca$^{2+}$ influx via SOC channels activates Ano1 channels. Store depletion and refill cycles in non-excitable cells typically occur on slow time-scales, on the order of tens of seconds, due to the time it takes for STIM1 to translocate to the plasma membrane (Edwards et al., 2010), whereas pacemaker activity in mouse small intestine ICC can have a period less than 2 s (Thomsen et al., 1998). However, rapid SOCE has been observed in skeletal muscle cells, in which a splice variant of STIM1 is permanently localised to the plasma membrane, allowing SOC channels to activate within milliseconds of depletion occurring (Darbellay et al., 2011; Edwards et al., 2010). Therefore, SOCE can function at the frequencies of Ca$^{2+}$ oscillations observed in ICC.

The inhibitory effects of cyclopiazonic acid, thapsigargin, and SK&F 96365 on ICC pacemaker activity suggest that SOCE plays a key role in ICC (Liu et al., 2005a; Torihashi et al., 2002). Although inhibiting SERCA pumps would be expected to inhibit Ca$^{2+}$ cycling regardless of whether SOCE was involved, Torihashi et al. (2002) demonstrated that the increase in [Ca$^{2+}$]$_i$ following SERCA inhibition was due to Ca$^{2+}$ influx rather than IP$_3$R-mediated Ca$^{2+}$ release. 2-APB, which has been routinely described as an IP$_3$R inhibitor in the ICC field (Lowie et al., 2011), is a more reliable inhibitor of SOCE than IP$_3$Rs (Bootman et al., 2002), lending further support to the hypothesis that SOC channels
are important for initiating the pacemaker cycle. Similarly, inhibition of pacemaker activity by Ni^{2+} is commonly used to indicate a role for T-type Ca^{2+} channels in ICC (Kim et al., 2002; Kito and Suzuki, 2003) but similar concentrations of Ni^{2+} also inhibit SOC channels (Flemming et al., 2003). The application of Ni^{2+} or Ca^{2+}-free external solution inhibited a Ca^{2+}-activated Cl\textsuperscript{−} current in ICC (Zhu et al., 2009), indicating that Ca^{2+} influx, rather than Ca^{2+} release, is important for activating Ano1 in ICC. Therefore, it is likely that Ano1 channels in ICC are colocalised with SOC channels.

Nevertheless, it is plausible that the Ca^{2+} pathway for Ano1 activation in ICC may instead be IP\textsubscript{3}R channels in sections of ER proximal to the plasma membrane. The lack of slow waves in IP\textsubscript{3}R1 knockout mice (Suzuki et al., 2000) and inhibition of slow wave activity when inhibiting IP\textsubscript{3} production with phospholipase C inhibitors (Lowie et al., 2011; Malysz et al., 2001) show that IP\textsubscript{3}Rs are a crucial component of the pacemaker Ca^{2+} cycle in ICC. In order for Ano1 to act as a pacemaker channel, a cyclical increase in [Ca^{2+}], of sufficient magnitude to activate Ano1 is required, but the source of Ca^{2+} does not affect the Ano1 model activation. Therefore, modelling the activation of Ano1 by IP\textsubscript{3}Rs localised near the cell membrane could be implemented with the same method that was used to model localisation of Ano1 to SOC channels in this study, with little impact on the rest of the model.

6.3.6 Conclusions

The model presented here is the first ICC model to incorporate Ano1 as the pacemaker channel. The model supports the hypothesis that Ano1 channels can act as a pacemaker channel in ICC in response to store-operated Ca^{2+} entry. Ano1 channels are necessary for the generation of slow waves in the model, in agreement with experimental observations (Hwang et al., 2009), but the Ano1 knockout simulations suggest that membrane potential oscillations are not completely abolished by blocking Ano1 currents.

The morphology of the simulated slow wave is highly dependent on E\textsubscript{Cl}, such that setting E\textsubscript{Cl} at the values measured by Zhu et al. (2010) in situ results in a shortened plateau phase. The microelectrode and patch-clamp recording techniques used to measure
membrane potentials may alter $E_{Cl}$, thereby altering the slow wave form, and experimental studies are needed to investigate this possibility.

The plateau current may be carried by a voltage- or $Ca^{2+}$-activated non-selective or $Na^{+}$ channel. Conversely, voltage-gated $Ca^{2+}$ currents are unlikely to contribute significantly to the plateau phase.

Future modelling studies can expand on the results presented here. Potential areas for future work include investigating the possible role of unitary potentials in the plateau phase in a model with Ano1 as the pacemaker channel, and exploring how slow wave propagation through an ICC network may depend on Ano1 currents.
Chapter 7

Conclusions and Future Directions

The work presented in this thesis has advanced our understanding of ICC function by comprehensively reviewing the possible mechanisms by which ICC may generate slow waves and developing models to better simulate ICC pacemaker activity. The primary contribution to the study of ICC pacemaker activity is the development of the first ICC model to incorporate Ano1 as a pacemaker channel. This is also the first ICC model to include SOCE as a component of the pacemaker mechanism. In addition, this thesis presents a pioneering model of ICC mechano-regulation, and an original model of Ano1 current. The following sections summarise the key findings and suggest important areas for future research.

7.1 The ICC Pacemaker Mechanism

A necessary step towards constructing a model of ICC pacemaker activity was assembling the available evidence on ion channels and Ca\(^{2+}\) dynamics in ICC into a pacemaker hypothesis. Therefore, a new hypothesis was developed to describe how ICC may generate slow waves. The proposed pacemaker cycle is initiated by IP\(_3\)R-mediated Ca\(^{2+}\) release from the ER, involves Ca\(^{2+}\)-dependent activation of a pacemaker channel, probably Ano1, and depends on T-type Ca\(^{2+}\) channels to activate during the upstroke phase, as detailed in Section 2.9.4. However, many aspects of the pacemaker mechanism need confirming, including the roles of SOCE and mitochondria in the Ca\(^{2+}\) cycle, and the exact roles
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of all the ion channels found in ICC. Furthermore, the link between different slow wave properties and differences in ion channel expression across species, organs, and classes of ICC needs to be better characterised.

Previously, the leading pacemaker hypothesis was the NSCC Hypothesis described in Section 2.9.1 (Sanders et al., 2006). This was the first comprehensive pacemaker hypothesis proposed in the literature but it was challenged by recent findings from experimental and modelling studies, which showed that the eponymous NSC channel was not likely to be inactive under resting conditions, and that an alternative pacemaker channel candidate—the Ano1 Cl\(^{-}\) channel—was essential for pacemaker activity in ICC (Hwang et al., 2009; Means and Sneyd, 2010). The NSCC Hypothesis formed the basis for the two most recent ICC models (Corrias and Buist, 2008; Faville et al., 2009). Taking into consideration the differences between the new pacemaker hypothesis and the existing ICC models, development of a novel ICC model was deemed necessary to test the new hypothesis and provide an updated model of pacemaker function (Section 3.4).

While reviewing the current state of knowledge of ICC research, one of the key themes that emerged was that limitations inherent in the experimental techniques used to study ICC make it difficult to ascertain the contribution of individual ion currents and Ca\(^{2+}\) fluxes to the pacemaker mechanism. Issues identified include: (i) the effects of recording techniques on ionic activity (Section 2.3.1), (ii) the use of cultured ICC as a primary model tissue (Section 2.3.2), and (iii) the use of pharmacological agents to characterise ion currents (Section 2.3.4). Firstly, both microelectrode impalement and whole-cell patch-clamp techniques can alter the intracellular ion concentrations, thereby altering the driving force and reversal potential of ion currents, as discussed in Section 6.3.2. Secondly, ICC change phenotype in culture (Epperson et al., 2000), yet cultured ICC were commonly used because of technical difficulties associated with the two alternatives: freshly dissociated ICC were difficult to identify prior to the advent of a GFP-Kit mouse model (Koh et al., 1998; Ro et al., 2010; Strege et al., 2003b), and recording directly from ICC in situ was only achieved in recent years (Wang et al., 2008a). Thirdly, many drugs used to identify Cl\(^{-}\) currents and Ca\(^{2+}\) fluxes in ICC have non-specific effects, making their effects on pacemaker activity difficult to interpret. These limitations may contribute
to the diversity and apparent inconsistencies of experimental results.

7.2 Modelling the Ano1 Channel

Ano1 was proposed as the candidate pacemaker channel in the new pacemaker hypothesis, so a novel model of the Ano1 channel was presented in Chapter 5. The Ano1 channel model reproduced the observed properties of steady-state Ano1 channel activation, with the highest open probability resulting from concurrently depolarised membrane potentials and high Ca\(^{2+}\) concentrations. The model also captured the kinetic behaviour of Ano1 channels, apart from the voltage-dependent deactivation of Ano1 channels in the presence of high [Ca\(^{2+}\)]\(_i\), which occurred more rapidly in the model than in Ano1 currents recorded by Xiao et al. (2011). The relatively slow kinetics of Ano1 in comparison to traditional voltage-gated ion channels and the synergistic effects of Ca\(^{2+}\) and membrane potential on open probability implied that activation of the Ano1 in an ICC model would be likely to require a prolonged, localised increase in [Ca\(^{2+}\)]\(_i\) in order to produce a significant current.

It is worth noting that multiple isoforms of Ano1 are expressed in human ICC (Mazzone et al., 2011), each with different kinetics and Ca\(^{2+}\) sensitivity (O’Driscoll et al., 2011). Customised models should be developed for each Ano1 isoform as quantitative electrophysiological data become available, so that the effects of variable isoform expression on slow wave generation can be explored in future modelling studies.

7.3 Modelling Slow Waves in ICC

The Ano1 model was incorporated into the ICC model described in Chapter 6. The ICC model presented in this thesis is the first ICC model to implement Ano1 as a pacemaker channel and incorporate SOCE. A series of model variations were developed, enabling several conclusions to be drawn: Firstly, the model supports the hypothesis that Ano1 can initiate slow waves in response to cyclical activation of SOC channels. The simulations reproduce the observation that blocking or knocking out Ano1 channels causes loss of slow wave activity. Secondly, E\(_{Cl}\) plays an important role in modulating the shape of the slow
wave. If $E_{Cl}$ is close to the potentials measured in ICC-MY by Zhu et al. (2010) then Ano1 current may contribute to repolarising the cell but the plateau phase is not well reproduced. Conversely, if $E_{Cl}$ is at least as high as the slow wave peak, membrane potential oscillations that recreate experimentally recorded slow waves can be easily simulated. It is possible that $E_{Cl}$ is artificially raised by the microelectrode recording techniques used to measure membrane potentials, such that recorded slow wave traces are different to those present in undisturbed ICC. Thirdly, voltage- or $Ca^{2+}$-activated non-selective channels or $Na^+$ channels are plausible candidates for generating the plateau current, but large or prolonged $Ca^{2+}$ currents are unlikely to contribute a major component of the slow wave depolarisation. Fourthly, membrane potential oscillations in ICC-MY from Ano1 knockout mice may not be completely abolished, but are minimised by background currents.

Further experiments are needed to confirm or refute these conclusions and to provide more evidence about the contributions of various ion channels to slow waves generated by pacemaker ICC in the GI tract. It is clear from the results presented in Chapter 6 and the literature reviewed in Chapter 2 that our understanding of how $Cl^-$ currents function in ICC is insufficient, and the study of native $Cl^-$ currents in ICC is limited by the pharmacological and electrophysiological techniques available. Nevertheless, it is particularly important to confirm the report of $[Cl^-]$ in ICC (Zhu et al., 2010) and to search for any evidence that the reversal potential of Ano1 currents differs from the expected value for $E_{Cl}$. Determining which ion channel or channels generate the plateau phase is also important, and future modelling studies can test the hypothesis that the plateau channel is colocalised with a $Ca^{2+}$ channel.

The primary purpose of the ICC model was to explore the sensitivity of the pacemaker mechanism in a single cell to changing ion currents and $E_{Cl}$. An important direction for future simulation studies will be investigating the propagation of slow waves in ICC networks and into smooth muscle tissue using an ICC model with Ano1 as the pacemaker channel.

All the ICC models developed to date, including the one presented in this thesis, have focused on the pacemaker function of ICC-MY in the stomach and small intestine. Another promising application of ICC modelling is to explore the function of intramuscular ICC,
which normally do not pace spontaneously, but can regenerate slow waves that originate in ICC-MY and can act as pacemaker cells under neural stimulation (Dickens et al., 2001; Hirst et al., 2002b). Modelling studies may aid in explaining which ion channels or Ca$^{2+}$ handling mechanisms underlie the functional differences between pacemaker ICC and intramuscular ICC.

## 7.4 Modelling ICC Mechanosensitivity

A new model of Na$^+$ channel mechanosensitivity was developed and incorporated into the Faville et al. (2009) small intestine ICC model to explore the effect of a mechanical stimulus on slow wave activity. During simulations of mechanical stimulation, Na$_V$1.5 mechanosensitivity was shown to increase multiple measures of ICC excitability, such as depolarising RMP, increasing upstroke rate, prolonging duration, and increasing frequency. These results agreed with the experimental effects of stretch on smooth muscle tissue, suggesting that Na$_V$1.5 channels are at least partly responsible for the experimentally observed regulation of slow waves in response to stretch.

The model of ICC mechanosensitivity presented in this thesis can be extended in several ways to provide additional insight into the interaction between mechanical and electrical signals in the GI tract. Multiscale models of ICC and SMC electrophysiology and excitation-contraction coupling will enable mechanoelectrical feedback to be studied (Du et al., 2011; Gajendiran and Buist, 2011). Crucially, the relationship between the patch pressure applied to the Na$_V$1.5 channels and the tension in smooth muscle tissue needs to be characterised to enable accurate modelling of mechanoelectrical feedback. Additional stretch-dependent elements can then be incorporated, such as other mechanosensitive ion channels in ICC, SMC, and neurons.

## 7.5 Concluding Remarks

The findings presented in this thesis confirm the complexity of the ICC pacemaker mechanism and highlight the importance of further research in this area. ICC models
7. CONCLUSIONS

are an important tool for understanding mechanisms by which ICC generate slow waves, and when integrated into multiscale models can be used to investigate how slow waves propagate and activate SMC. However, models are limited by the available information on ion channels and processes in ICC. Currently, more experimental research is needed to address the questions raised by the work presented here. As our understanding of ICC electrophysiology progresses and more models are developed, a diversity of ICC models may elucidate the differences between pacemaker mechanisms in different regions of the GI tract and across different species. Much is still unknown about the enigmatic ICC, and they will remain a fertile area of research for many years to come.
Appendix A

Publications and Awards

A.1 Journal Publications


A.2 Book Chapter


A.3 Conference Abstracts


A.4 Awards and Scholarships


2. Winter School in Mathematical and Computational Biology Travel Bursary, Brisbane, QLD, Australia, 2012.

3. AGA-Horizon Pharma Student Abstract Prize, Digestive Disease Week, Chicago, IL, USA, 2011.
Appendix B

ICC Model Equations and Parameters

Appendix B lists the equations used in the ICC model from Chapter 6. Parameter values common to all model variations are given in Table B.1, while parameter values and gating variables that differ across the model variations are in Tables B.2 and B.3, respectively.

B.1 State Variables

Membrane Potential

\[
\frac{dV_m}{dt} = -\frac{I_{ion}}{C_m} \tag{B.1}
\]

where \(I_{ion}\) for each model variation is defined in Equations 6.3–6.6, 6.8, and 6.9.

Ion Channel Gating Variables

\[
\frac{dx}{dt} = \frac{x_\infty - x}{\tau_x} \tag{B.2}
\]

for \(x = O_{Ano1}, d_{CaT}, f_{CaT}, d_{NaV}, f_{NaV}, d_{CaV}, d_{NSV}, f_{NSV}, d_{Kt}, f_{Kt}, d_{KERG},\) or \(f_{KERG}\). Parameter values for \(x_\infty\) and \(\tau_x\) are listed in Table B.3, except for \(O_{Ano1}\) given in Equations B.14 and B.16 respectively, \(\tau_{d_{NaV}}\) given in Equations B.31 and B.37, and \(\tau_{d_{KERG}}\) given in Equation B.40.
Table B.1: Parameter values common to all ICC model variations. C denotes that the parameter value was calculated using other parameters. References are given where available; other parameter values were chosen to reproduce correct slow wave activity.

*IP$_3$R $k_i$ values are double the values in Wang et al. (2010a) in order to reproduce correct Ca$^{2+}$ oscillation frequency.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
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</tr>
</thead>
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<tr>
<td><strong>Cell constants</strong></td>
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<td></td>
</tr>
<tr>
<td>$C_m$</td>
<td>25 pF</td>
<td>(Goto et al., 2004; Kim et al., 2002)</td>
</tr>
<tr>
<td>$v$</td>
<td>$1 \times 10^{-12}$ L</td>
<td>(Corrias and Buist, 2008)</td>
</tr>
<tr>
<td>$v_{cyto}$</td>
<td>$0.7 \times 10^{-12}$ L</td>
<td>(Corrias and Buist, 2008)</td>
</tr>
<tr>
<td>$v_{er}$</td>
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<td>(Corrias and Buist, 2008)</td>
</tr>
<tr>
<td>$F$</td>
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<td></td>
</tr>
<tr>
<td>$R$</td>
<td>8.314 J mol$^{-1}$ K$^{-1}$</td>
<td></td>
</tr>
<tr>
<td>$T$</td>
<td>310.0 K</td>
<td></td>
</tr>
<tr>
<td><strong>Plasma membrane current parameters</strong></td>
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<td></td>
</tr>
<tr>
<td>$[\text{Ca}^{2+}]_o$</td>
<td>2 mM</td>
<td>(Corrias and Buist, 2008; Faville et al., 2009)</td>
</tr>
<tr>
<td>$[\text{Cl}^-]_o$</td>
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<td>(Zhu et al., 2010)</td>
</tr>
<tr>
<td>$[\text{K}^+]_i$</td>
<td>140 mM</td>
<td>(White et al., 2008)</td>
</tr>
<tr>
<td>$[\text{K}^+]_o$</td>
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<td>(White et al., 2008)</td>
</tr>
<tr>
<td>$[\text{Na}^+]_i$</td>
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<td>(Corrias and Buist, 2008)</td>
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<tr>
<td>$[\text{Na}^+]_o$</td>
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<td>(Corrias and Buist, 2008)</td>
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<td><strong>Cytosolic Ca$^{2+}$ flux parameters</strong></td>
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</tr>
<tr>
<td>$K_{SOC}$</td>
<td>200 µM</td>
<td>(Brandman et al., 2007; Stathopulos et al., 2008)</td>
</tr>
<tr>
<td>$K_{PMCA}$</td>
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<td></td>
</tr>
<tr>
<td>$b_c$</td>
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<td>(Fall and Keizer, 2001)</td>
</tr>
<tr>
<td><strong>IP$_3$R parameters</strong></td>
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<td></td>
</tr>
<tr>
<td>$P$</td>
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<td>(Wang et al., 2010a)</td>
</tr>
<tr>
<td>$k_1$</td>
<td>4000 µM$^{-1}$ s$^{-1}$</td>
<td>(Wang et al., 2010a)*</td>
</tr>
<tr>
<td>$k_2$</td>
<td>2.0 µM$^{-1}$ s$^{-1}$</td>
<td>(Wang et al., 2010a)*</td>
</tr>
<tr>
<td>$k_3$</td>
<td>4000 µM$^{-1}$ s$^{-1}$</td>
<td>(Wang et al., 2010a)*</td>
</tr>
<tr>
<td>$k_4$</td>
<td>2.0 µM$^{-1}$ s$^{-1}$</td>
<td>(Wang et al., 2010a)*</td>
</tr>
<tr>
<td>$k_5$</td>
<td>200 µM$^{-1}$ s$^{-1}$</td>
<td>(Wang et al., 2010a)*</td>
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<td>520 s$^{-1}$</td>
<td>(Wang et al., 2010a)*</td>
</tr>
<tr>
<td>$k_{-2}$</td>
<td>2.1 s$^{-1}$</td>
<td>(Wang et al., 2010a)*</td>
</tr>
<tr>
<td>$k_{-3}$</td>
<td>3772 s$^{-1}$</td>
<td>(Wang et al., 2010a)*</td>
</tr>
<tr>
<td>$k_{-4}$</td>
<td>0.29 s$^{-1}$</td>
<td>(Wang et al., 2010a)*</td>
</tr>
<tr>
<td>$k_{-5}$</td>
<td>16.4 s$^{-1}$</td>
<td>(Wang et al., 2010a)*</td>
</tr>
<tr>
<td>$k_{IPR}$</td>
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</tr>
<tr>
<td>$J_{leak}$</td>
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Table B.1: (Continued)

<table>
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<td>$V_e$</td>
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</tr>
<tr>
<td>$K_e$</td>
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<td>(Wang et al., 2010a)</td>
</tr>
<tr>
<td><strong>Ano1 model parameters</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$E_{C50(0mV)}$</td>
<td>$1.39\mu\text{M}$</td>
<td>(Xiao et al., 2011)</td>
</tr>
<tr>
<td>$k_c$</td>
<td>$0.01248 \text{mV}^{-1}$</td>
<td>(Xiao et al., 2011)</td>
</tr>
<tr>
<td>$V_h$</td>
<td>$-100 \text{mV}$</td>
<td></td>
</tr>
<tr>
<td>$k_v$</td>
<td>$0.0156 \text{mV}^{-1}$</td>
<td>(Xiao et al., 2011)</td>
</tr>
<tr>
<td><strong>Ano1-SOC localisation parameters</strong></td>
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<td></td>
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<tr>
<td>$D_c$</td>
<td>$250 \mu\text{m}^2 \text{s}^{-1}$</td>
<td>(Smith, 1996)</td>
</tr>
<tr>
<td>$D_m$</td>
<td>$75 \mu\text{m}^2 \text{s}^{-1}$</td>
<td>(Smith, 1996)</td>
</tr>
<tr>
<td>$K_m$</td>
<td>$1 \mu\text{M}$</td>
<td>(Smith, 1996)</td>
</tr>
<tr>
<td>$B_m$</td>
<td>$50 \mu\text{M}$</td>
<td>(Smith, 1996)</td>
</tr>
<tr>
<td>$r$</td>
<td>$0.05 \mu\text{m}$</td>
<td></td>
</tr>
<tr>
<td>$v_{\text{scale}}$</td>
<td>$10^{15} \mu\text{m}^3 \text{L}^{-1}$</td>
<td>C</td>
</tr>
<tr>
<td>$n_{SOC}$</td>
<td>$50$</td>
<td>(Faville et al., 2009)</td>
</tr>
</tbody>
</table>

**Calcium Concentration**

The change in $[\text{Ca}^{2+}]_{\text{ER}}$ is given by:

$$\frac{d[\text{Ca}^{2+}]_{\text{ER}}}{dt} = (J_{\text{SERCA}} - J_{\text{IPR}}) \frac{v_{\text{cyto}}}{v_{\text{ER}}}$$  \hspace{1cm} (B.3)

The change in $[\text{Ca}^{2+}]_i$ is given by:

$$\frac{d[\text{Ca}^{2+}]_i}{dt} = b_c (J_{\text{IPR}} - J_{\text{SERCA}} + J_{\text{SOC}} + J_{\text{CaT}} - J_{\text{PMCA}})$$  \hspace{1cm} (B.4)

as in Equation 6.2, except in High-Cl(CaV) and Low-Cl(NSCa) where it is replaced by Equations 6.7 and 6.10 respectively.

**IP$_3$R Inactivation**

The proportion of IP$_3$R channels inactivated by $\text{Ca}^{2+}$ ($y$), as a function of $\text{Ca}_i$ and IP$_3$ concentration ($P$), from Wang et al. (2010a):

$$\frac{dy}{dt} = \phi_1 (1 - y) - \phi_2 y$$  \hspace{1cm} (B.5)
B. ICC MODEL EQUATIONS AND PARAMETERS

\[
\phi_1 = \frac{(k_{-4}K_2K_1 + k_{-2}K_4P)Ca_i}{K_4K_2(K_1 + P)} \quad \text{(B.6)}
\]

\[
\phi_2 = \frac{k_{-2}P + k_{-4}K_3}{K_3 + P} \quad \text{(B.7)}
\]

\[
K_i = \frac{k_{-i}}{k_i} \quad \text{(B.8)}
\]

B.2 Calcium Flux

Ca\(^{2+}\) Flux Across the Plasma Membrane

Plasma membrane Ca\(^{2+}\) ATPase:

\[
J_{\text{PMCA}} = \frac{V_{\text{PMCA}}Ca_i^2}{K_{\text{PMCA}}^2 + Ca_i^2} \quad \text{(B.9)}
\]

Na\(^+\)/Ca\(^{2+}\) exchanger, from Livshitz et al. (2012):

\[
J_{\text{NCX}} = \frac{V_{\text{NCX}}Ca_i^3}{3.6 + Ca_i} \quad \text{(B.10)}
\]

Ca\(^{2+}\) Flux Across the ER Membrane

Total Ca\(^{2+}\) release from the ER, adapted from Wang et al. (2010a):

\[
J_{\text{IPR}} = (k_{\text{IPR}}O_{\text{IPR}} + J_{\text{leak}})(Ca_{\text{ER}} - Ca_i) \quad \text{(B.11)}
\]

\[
O_{\text{IPR}} = \left( \frac{Ca_iP(1 - y)}{(P + K_1)(Ca_i + K_5)} \right)^3 \quad \text{(B.12)}
\]

Ca\(^{2+}\) uptake into the ER, from Wang et al. (2010a):

\[
J_{\text{SERCA}} = \frac{V_eCa_i^2}{K_e^2 + Ca_i^2} \quad \text{(B.13)}
\]

B.3 Ano1 model

The equations for the Ano1 model from Chapter 5 are detailed in Section B.3.
**Steady-State Activation**

Steady-state open probability of the Ano1 channel model, developed using data from (Xiao et al., 2011):

\[
O_{\text{Ano1}\infty} = \frac{1}{1 + e^{(V_h - V_m)k_e}} \left( 1 + \left( \frac{EC_{50}}{Ca_{\text{Ano1}}} \right)^2 \right),
\]

(B.14)

\[
EC_{50} = EC_{50(0mV)} e^{-k_c V_m}.
\]

(B.15)

**Activation Kinetics**

Time constant calculation for the Ano1 channel model, developed using data from (Xiao et al., 2011):

\[
\tau_{\text{Ano1}} = t_1 + t_2 e^{V_m/t_3}
\]

(B.16)

\[
t_1 = 0.08163 e^{-0.57 Ca_{\text{Ano1}}}
\]

(B.17)

\[
t_2 = 0.07617 e^{-0.05374 Ca_{\text{Ano1}}}
\]

(B.18)

\[
t_3 = 70.3 e^{0.153 Ca_{\text{Ano1}}}
\]

(B.19)

**B.4 Ano1-SOC Localisation**

\(Ca^{2+}\) concentration near Ano1 channels as a function of \(Ca^{2+}\) flux through SOC channels, from Smith (1996):

\[
Ca_{\text{Ano1}} = \left( -D_c K_m + \frac{\sigma}{2\pi r} + C_2 + \sqrt{\left( D_c K_m + \frac{\sigma}{2\pi r} + C_2 \right)^2 + 4D_c \Phi_m} \right) / 2D_c
\]

(B.20)

\[
C_2 = D_c C_{ai} - \frac{\Phi_m}{K_m + C_{ai}}
\]

(B.21)

\[
\Phi_m = D_m B_m K_m
\]

(B.22)

\[
\sigma = \frac{v_{scale} J_{\text{SOC}} v_{\text{cyto}}}{n_{\text{SOC}}}
\]

(B.23)
B. ICC MODEL EQUATIONS AND PARAMETERS

Table B.2: Parameter values that differ across ICC model variations. References are given where available; the remaining parameter values were calculated using other parameters (C) or chosen to reproduce correct slow wave activity. Model variations in which each parameter value is used are denoted by H for High-Cl and L for Low-Cl.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Reference</th>
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</thead>
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<tr>
<td><strong>Cl⁻ parameters</strong></td>
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</tr>
<tr>
<td>[Cl⁻]ᵢ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>78 mM</td>
<td>C</td>
</tr>
<tr>
<td>L</td>
<td>25.85 mM</td>
<td>(Zhu et al., 2010)</td>
</tr>
<tr>
<td>E₈₀</td>
<td></td>
<td></td>
</tr>
<tr>
<td>H</td>
<td>-20.2 mV</td>
<td>C</td>
</tr>
<tr>
<td>L</td>
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<td><strong>Ion channel maximal conductances</strong></td>
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<tr>
<td>g_{Ano1}</td>
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<tr>
<td>H(NSV,NSCa)</td>
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<tr>
<td>H(NaV,CaV), L(NaV)</td>
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</tr>
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<td>L(NSCa)</td>
<td>10 nS</td>
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<tr>
<td>g_{CaT}</td>
<td></td>
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<tr>
<td>H(NaV,NSV,CaV), L(NaV)</td>
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<td>(Corrias and Buist, 2008)</td>
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<td>H(NSCa), L(NSCa)</td>
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<td>g_{Nab}</td>
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<td>g_{NSCa}</td>
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<td>g_{Kt}</td>
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<td>g_{KERG}</td>
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<td><strong>Ca²⁺ pump maximal fluxes</strong></td>
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<td>V_{PMCA}</td>
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<td>L(NSCa)</td>
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Table B.3: Parameters for voltage-dependent gating variables for $x_\infty = a/\left(1 + e^{(V_m-V_h)/k}\right) + (1-a)$ and for $\tau_x$, where $x = d_{CaT}, f_{CaT}, d_{NaV}, f_{NaV}, d_{CaV}, d_{NSV}, f_{NSV}, d_{Kt}, f_{Kt}, d_{KERG}$, or $f_{KERG}$. Model variations in which each parameter value is used are denoted by H for High-Cl and L for Low-Cl. References are given where available; other parameter values were chosen to reproduce correct slow wave activity. *$V_h$ modified from values in Beyder et al. (2010).

<table>
<thead>
<tr>
<th>Variable</th>
<th>$V_h$ (mV)</th>
<th>$k$ (mV)</th>
<th>$a$</th>
<th>$\tau_x$ (s)</th>
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<td>26</td>
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<td>(Corrias and Buist, 2008)</td>
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<td>-40</td>
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<td>(Beyder et al., 2010)*</td>
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<td>(Beyder et al., 2010)</td>
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B. ICC MODEL EQUATIONS AND PARAMETERS

B.5 Ion Currents

Ion Currents Common to All Models

Store-operated Ca\(^{2+}\) channel:

\[
I_{SOC} = g_{SOC} \cdot O_{SOC} (V_m - E_{Ca})
\] (B.24)

\[
O_{SOC} = \frac{K_{SOC}^8}{K_{SOC}^8 + Ca_{ER}^8}
\] (B.25)

Ano1 Ca\(^{2+}\)-activated Cl\(^{-}\) channel:

\[
I_{Ano1} = g_{Ano1} \cdot O_{Ano1} (V_m - E_{Cl})
\] (B.26)

Na\(^{+}\) background current:

\[
I_{Nab} = g_{Nab} (V_m - E_{Na})
\] (B.27)

K\(^{+}\) background current:

\[
I_{Kb} = g_{Kb} (V_m - E_{K})
\] (B.28)

T-type Ca\(^{2+}\) current:

\[
I_{CaT} = g_{CaT} \cdot d_{CaT} \cdot f_{CaT} (V_m - E_{Ca})
\] (B.29)

Ion Current Specific to High-Cl(NaV)

Voltage-gated Na\(^{+}\) channel:

\[
I_{NaV} = g_{NaV} \cdot d_{NaV} (V_m - E_{Na})
\] (B.30)

\[
\tau_{dNaV} = \left(50 + \frac{1000}{1 + e^{-0.06(V_m + 20)}}\right)^{-1}
\] (B.31)
B.5. Ion Currents

**Ion Current Specific to High-Cl(NSV)**

Voltage-gated non-selective channel:

\[ I_{NSV} = g_{NSV} \cdot d_{NSV} \cdot f_{NSV} \left( V_m - E_{NS} \right) \]  

(B.32)

**Ion Current Specific to High-Cl(NSCa)**

Ca\(^{2+}\)-activated non-selective channel:

\[ I_{NSCa} = g_{NSCa} \cdot O_{NSCa} \left( V_m - E_{NS} \right) \]  

(B.33)

\[ O_{NSCa} = \frac{Ca_i^4}{1.84 + Ca_i^4} \]  

(B.34)

**Ion Current Specific to High-Cl(CaV)**

Voltage-gated Ca\(^{2+}\) channel:

\[ I_{CaV} = g_{CaV} \cdot d_{CaV} \left( V_m - E_{Ca} \right) \]  

(B.35)

**Ion Currents Specific to Low-Cl(NaV)**

Voltage-gated Na\(^{+}\) channel:

\[ I_{NaV} = g_{NaV} \cdot d_{NaV} \cdot f_{NaV} \left( V_m - E_{Na} \right) \]  

(B.36)

\[ \tau_{dNaV} = \left( 50 + \frac{1000}{1 + e^{-0.06(V_m+20)}} \right)^{-1} \]  

(B.37)

Transient K\(^{+}\) channel:

\[ I_{Kt} = g_{Kt} \cdot d_{Kt} \cdot f_{Kt} \left( V_m - E_{K} \right) \]  

(B.38)
B. ICC MODEL EQUATIONS AND PARAMETERS

ERG-like $K^+$ channel:

$$I_{K_{ERG}} = g_{K_{ERG}} \cdot d_{K_{ERG}} \cdot f_{K_{ERG}} (V_m - E_K) \quad (B.39)$$

$$\tau_{d_{K_{ERG}}} = \left( 1 + \frac{1}{1 + e^{0.15(V_m + 60)}} \right)^{-1} \quad (B.40)$$

**Ion Currents Specific to Low-Cl(NSCa)**

$Ca^{2+}$-activated non-selective channel:

$$I_{NSCa} = g_{NSCa} \cdot O_{NSCa} (V_m - E_{NS}) \quad (B.41)$$

$$O_{NSCa} = \frac{Ca_i^5}{1.3^5 + Ca_i^5} \quad (B.42)$$

$Na^+/Ca^{2+}$ exchanger, from Livshitz et al. (2012):

$$I_{NCX} = -2F \cdot J_{NCX} \cdot v_{cyto} \quad (B.43)$$

Nernst Equilibrium Potential

$$E_{Ca} = \frac{RT}{2F} \ln \left( \frac{[Ca^{2+}]_o}{[Ca^{2+}]_i} \right) \quad (B.44)$$

$$E_{Cl} = \frac{RT}{F} \ln \left( \frac{[Cl^-]_i}{[Cl^-]_o} \right) \quad (B.45)$$
References


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