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CELLULAR ORIGIN AND REGULATION OF
THE ELECTRICAL SLOW WAVE
IN THE MURINE SMALL INTESTINAL MUSCULATURE

by

JOHN MALYSZ, B.SC.

A Thesis
Submitted to the School of Graduate Studies
in Partial Fulfillment of the Requirements
for the Degree of Ph.D. in
Medical Sciences

McMaster University

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CELLULAR ORIGIN AND REGULATION OF
THE ELECTRICAL SLOW WAVE
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Abstract

The electrical pacemaker slow wave is responsible for the generation of anally propagating phasic contractions underlying the peristaltic motor activity of the gastrointestinal musculature. Yet, the cellular origins of the slow wave and mechanisms of the slow wave regulation or generation still remain unresolved and constituted primary goals of the current thesis. As described in detail in Chapters Three – Six, spontaneously genetic knock out mice with genetic mutations affecting the structure ($W/W^m$ mice), expression ($W^{rd}/W^{rd}$ mice), or the ligand ($Sl/Si$ mice) of the kit tyrosine kinase receptor were shown to lack both the network of interstitial cells of Cajal associated with the myenteric plexus and the slow wave activity in the small intestine, hence, supporting the proposed role of the interstitial cells of Cajal as pacemaker cells responsible for the slow wave generation. In the absence of the slow wave, the mutant musculature was either electrically quiescent or showed action potentials in regular or irregular patterns as recorded with a standard microelectrode technique. The observed action potentials were also clearly distinguished from the slow waves by their shape and pharmacological sensitivities to L-type Ca$^{2+}$ channel and K$^+$ channel blockade.

The mechanisms of the slow wave generation and regulation are addressed in Chapters Seven – Nine. The data indicate that the slow wave generation involves primarily Na$^+$ and Ca$^{2+}$ conductances not mediated by TTX- or mexiletine-sensitive Na$^+$ channels, gadolinium sensitive nonselective cation channels, or L-type Ca$^{2+}$ channels. Cl$^-$
channels may be also involved in the regulation but not in the slow wave initiation. Pharmacological agents acting on cytosolic Ca\textsuperscript{2+}, SR Ca\textsuperscript{2+} ATPase, and intracellular Ca\textsuperscript{2+} release mechanisms support the role of intracellular Ca\textsuperscript{2+} release mechanisms, sensitive to IP\textsubscript{3}, in the regulation of the slow wave frequency and amplitude. Furthermore, activation of the Ca\textsuperscript{2+} induced Ca\textsuperscript{2+} release (CICR) mechanism leads to depolarization not mediated predominantly by chloride channels nor likely by K\textsubscript{Ca} channels. The CICR may be also involved in the regulation of the slow wave. These experiments importantly identify intracellular metabolic pathways that may potentially lead to the development of therapeutic approaches aimed at treating certain gastrointestinal motor disorders by modifying the slow wave frequency or amplitude.
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Finally, I dedicate this thesis to my wife, Joanna, for her love, understanding, support and encouragement that enabled me to undertake the effort to complete this project. I am also grateful to my parents, brothers, and sister.

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<tr>
<td>AP</td>
<td>Auerbach plexus</td>
</tr>
<tr>
<td>2APB</td>
<td>2-aminoethoxydiphenyl borate</td>
</tr>
<tr>
<td>4-AP</td>
<td>4-aminopyridine</td>
</tr>
<tr>
<td>BAPTA/AM</td>
<td>bis-(o-aminophenoxy)-ethane-N,N,N',N'-tetra-acetic, tetra (acetoxyethyl)-ester</td>
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<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CPA</td>
<td>Cyclopiazonic acid</td>
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<tr>
<td>CICR</td>
<td>Calcium induced calcium release</td>
</tr>
<tr>
<td>D600</td>
<td>Methoxyverapamil</td>
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<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
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<tr>
<td>DIDS</td>
<td>4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid</td>
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<td>DMP</td>
<td>Deep muscular plexus</td>
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<tr>
<td>E&lt;sub&gt;Cl&lt;/sub&gt;</td>
<td>Nernst equilibrium potential for chloride</td>
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<td>FLC</td>
<td>Fibroblast-like cell</td>
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<td>IBMX</td>
<td>3-isobutyl-1-methyl-xanthine</td>
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<td>ICC</td>
<td>Interstitial cell of Cajal</td>
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<td>Inositol 1,4,5-trisphosphate</td>
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$K_{\text{ATP}}$  ATP sensitive $K^+$ channel

$K_{\text{Ca}}$  Calcium activated $K^+$ channel

$K_{\text{DR}}$  Delayed rectifier $K^+$ channel

$M_3$  Muscarinic type 3 receptor

$\text{MLC}$  Macrophage like cell

$\text{NCDC}$  2-nitro-4-carboxylphenyl-N,N-diphenylcarbamate

$\text{PKA}$  Protein kinase A

$\text{PKC}$  Protein kinase C

$\text{PKG}$  Protein kinase G

$\text{PLC}$  Phospholipase C

$\text{RyR}$  Ryanodine receptor

$\text{SMC}$  Smooth muscle cell

$\text{SNP}$  Sodium nitroprusside

$S/l$  Steel factor gene locus

$\text{SR}$  Sarcoplasmic reticulum

$\text{TEA}$  tetrathylammonium chloride

$W$  White spotting gene locus
Chapter One

Introduction

The small intestine extending from the stomach to the colon arbitrarily includes the duodenum, jejunum, and ileum. The three parts respectively occupy ~ 5, 40, and 55 % of the total length, ~ 6-7 m in an average adult human (Granger et al. 1985). Major functions of the organ are digestion and absorption of nutrients (carbohydrates, proteins and fats), secretion of hormones, and participation in the immune responses (Granger et al. 1985; Wingate, 1983). In order to maximize digestion and absorption, intestinal contents are mixed and propelled in the net aboral direction through a series of motor patterns generated by the small intestinal musculature. Even though the musculature consists of two separate muscle layers (longitudinal and circular) oriented roughly orthogonally to each other, the intestinal motor movements involve a high degree of coordination of the two muscle layers. For example, in peristalsis aborally propagating series of waves of contractions are observed. Regulation of the motor patterns involves neurogenic and myogenic control mechanisms. The electrical activities of the musculature, in particular the electrical slow waves, comprise the latter control mechanism and are discussed in detail below.
Structure of the small intestine

The small intestine forms a tubular structure. At every level of the small intestinal wall, clear partitions into layers are observed (figure 1) (Granger et al. 1985; Junqueira et al. 1992). The outermost layer, serosa, consists of simple squamous epithelium. The next layer, muscularis externa, is made up two discrete layers of smooth muscle cells: the outer longitudinal and the inner circular muscle layers. Within each layer orientation of the smooth muscle cells differs. Cells in the longitudinal layer are aligned in the longitudinal or long axis direction and those in the circular layer along the circumference of the wall. The well-defined separation of the two muscle layers occurs along a connective tissue septum within which neural processes and cell bodies forming the myenteric or Auerbach plexus, are found. Associated with the myenteric plexus is a network of small cells known as interstitial cells of Cajal (ICC) (Thuneberg, 1989; Faussone-Pellegrini, 1992). ICC have a characteristic morphology consisting of triangular, stellate cell bodies with thin branching cytoplasmic extensions or processes. Another location that houses a network of ICC in the small intestine is within the inner circular muscle layer at the subdivision of the circular muscle layer into the outer and inner circular muscle layers (Thuneberg, 1989; Faussone-Pellegrini, 1992). This location also contains neural processes or fibers forming the deep muscular plexus. As a result, ICC found at this location are described as being associated with the deep muscular plexus. The two other innermost layers of the small intestine are submucosa and mucosa. The former layer consists of connective tissue, blood vessels, lymphatics, ganglion cells and nerve fibers of the Meissner’s plexus. The mucosal layer is made up of a continuous
Figure 1. Organization of layers in the small intestine. Interstitial cells of Cajal are found as networks of cells associated with the myenteric or Auerbach plexus and with the deep muscular plexus. An example of the network of ICC in tissue from the murine small intestine is shown in figure 1(a) of Chapter Three. Adapted from Rumessen and Thuneberg (1996).
sheet of epithelial cells within which goblet cells, enterocytes, endocrine cells, M cells, intraepithelial lymphocytes, and Paneth cells are found. These cells are responsible for the functions of mucosa in digestion, absorption, mucus secretion, hormone secretion, and absorption of food antigens (Granger et al. 1985; Junqueira et al. 1992).

**Electrical activities of the intestinal musculature**

The gut musculature generates two types of electrical activities: slow waves and action potentials; both play different physiological roles. In the past, slow waves have been also referred to as action currents, basic electrical rhythms, pacesetter potentials, first potentials, and electrical control activities (Tomita, 1981; Szurszewski, 1997). The term, slow wave, refers to a continuously generated, periodic oscillation of the cell membrane potential with repeating periods of high and low membrane excitability (figure 2) (Huizinga, 1991; Tomita, 1981). On the plateau phase of the slow wave or during the high excitability phase, the membrane potential is brought very close to the level required for the activation of voltage sensitive ion channels responsible for the generation of action potentials. In contrast, in between the slow wave cycles or during the low excitability phase, the action potentials are not usually generated due to unfavorable conditions for the activation of the voltage sensitive ion channels. A unique feature of the slow wave is that it has a characteristic frequency depending on the animal species and organ. For example in the proximal murine small intestine and human antrum, the frequencies are \( \sim 48 \text{ cpm} \) (Der-Silaphet et al. 1998) and \( \sim 4 \text{ cpm} \) (El-Sharkawy et al. 1978), respectively.
Figure 2. Relationship among slow waves, action potentials and contractions, see text for discussion. Based on data obtained from the canine colon. Adapted from Huizinga et al. (1997).
One type of a voltage sensitive ion channel involved in action potential generation is the \( \text{L-type Ca}^{2+} \) channel (Huizinga, 1991). Opening of this class of \( \text{Ca}^{2+} \) channels allows for influx of \( \text{Ca}^{2+} \), which subsequently initiates the contractile responses of the smooth muscle cells. Hence, a close relationship exists between the intensity of action potentials (amplitude and frequency) and the force of contractions (figure 2). Furthermore, since action potential generation is usually restricted to the plateau phases of the slow waves, the highest force of contraction develops during the plateaus. As illustrated in figure 2, the slow waves do not necessarily lead to contractions. This is because the membrane potential that is reached on the plateau phase of the slow wave falls usually just short of the threshold level being required for the action potential generation. This threshold level is reached when smooth muscle cells sufficiently depolarize, as for example following gut wall distention. In this case, the gut wall distention triggers neuronal circuitry, the net result being the release of excitatory neurotransmitters (e.g. acetylcholine) in the proximity of smooth muscle cells as well as the removal of inhibitory control exerted on smooth muscle cells by the non-adrenergic, non-cholinergic inhibitory neurons (Wingate, 1983).

**Historical background**

At the turn of the century, nearly 100 years ago, Bayliss and Starling identified peristaltic movements of the canine small intestine in detail (Bayliss and Starling, 1899). Cannon's work utilizing for the first time the newly invented x-rays led to detailed descriptions of esophageal, gastric and bowel contractions (Cannon and Moser, 1898;
Cannon, 1902). More elaborate accounts of the in vivo bowel movements were provided by Code and his coworkers (Carlson et al. 1966). Their work with improved x-ray technology gave detailed explanations of the terminal antral contractions and retropulsion, rhythmic segmenting contractions, and peristaltic sweeps. Code’s other contribution was the introduction of methods that allowed simultaneous recording of the bowel electrical activity with the contractile pattern.

The first electrical recordings of the slow wave were made with extracellular electrodes. In 1907, Marimon identified slow waves in the chicken gizzard (Marimon, 1907). Later in 1922, Alvarez distinguished this type of electrical activity, which he originally defined as action currents (now referred to as slow waves), in a patient with a thin abdomen when electrodes were placed below the navel (Alvarez, 1922). In the 1930’s, Berkson investigated the nature of the action currents. He identified that action currents were associated only periodically with contractions, that they were independent of the extrinsic nervous system (Berkson, 1932; Berkson et al. 1932), and he proposed that that action currents originated from within ‘the intrinsic nervous plexus’ (Berkson, 1933; Berkson, 1932). This proposal was incorrect since the slow wave generation does not involve the enteric neurons (see below).

Extracellular recording methods also revealed the presence of fast spike shaped action potentials as reported by Bozler (1942) and Ambache (1947) in the small intestines of guinea pigs, rabbits, cats, and dogs. Bozler’s other contributions were on quantitative analyses showing that visceral smooth muscles are electrically interconnected forming a functional syncytium and that propagation of the slow waves occurs via smooth muscle
cell bundles (Bozler, 1938). Intracellular recording methods were introduced in the field of gut physiology in the 1950's. Initial studies were conducted on the guinea pig taenia coli by Bulbring (1954). Daniel's work on the canine small intestine (Daniel et al. 1959) and Prosser's work on the feline stomach (Papasova et al. 1968) and small intestine (Prosser and Sperelakis, 1956; Liu et al. 1969; Kobayashi et al. 1967) followed. It is worthwhile noting that in contrast to the guinea pig taenia coli, the antrum and the small intestine show omnipresent rhythmic slow waves. The current focus has been aimed at the slow wave, at understanding its ionic basis, synchronization and propagation as well as at identification of the pacemaker sites responsible for its generation. In fact, one of the major contributions of Prosser was to show that slow wave generation and conduction were nerve independent (Prosser and Sperelakis, 1956). Hence, this observation defeated a dominating hypothesis at the time considering neurons of the myenteric plexus to be responsible for the slow wave generation. Originally, Prosser viewed the longitudinal smooth muscle cells in the small intestine as the pacemaker cells (Connor et al. 1977). Now, we know that the pacemaker region in the small intestine is located between the longitudinal and circular muscle layers in the area associated with the myenteric plexus, not in the longitudinal muscle layer. This notion was originally considered by Kobayashi et al. (1967) for the feline small intestine and latter by Cheung and Daniel et al. (1980) for the rabbit small intestine.

Until 1982, smooth muscle cells were still erroneously thought to be responsible for the initiation of the slow wave. In 1982, Thuneberg for the first time provided physiological evidence suggesting that ICC located between the circular and longitudinal
muscle layers in the small intestine are responsible for the slow wave formation. In particular, he showed that perfusion of murine small intestinal muscle strips with methylene blue led to the accumulation of this agent selectively in ICC causing lesion of these cells and abolition of the slow wave (Thuneberg, 1982). His additional contribution involved compilation of an extensive ultrastructural description of various types of ICC that is still used today as a blueprint for the identification of these cells (Thuneberg, 1989). It is worthwhile noting that prior to 1982, other investigators also speculated that ICC may play a pacemaker role. In 1924, Tiegs proposed that ICC were involved in coordination of rhythmic contractions (Tiegs, 1924). Later in 1947, Ambache speculated that the slow wave origin and conduction involves the network of ICC (Ambache, 1947). A similar conclusion was also reached by Faussone-Pellegrini (1977).

The intrinsic slow wave frequency gradient plays a critical role in determining the direction of peristalsis. Since the intrinsic frequency gradient decreases along the length of the small bowel in the aboral direction, the direction of the contractions underlying peristaltic waves is also in the aboral direction (see next section for detailed explanation). The first evidence for the presence of the gradient was provided by Alvarez in 1914 while studying isolated and intact pieces of the rabbit small intestine (Alvarez, 1914). Further studies by Alvarez and co-workers showed that the slow wave frequency gradient and the maximal frequency of rhythmic contractions were identical (Alvarez and Mahoney, 1922). This observation formed a basis for the current, still-valid concept that slow waves provide a mechanism by which the gut motility is regulated and controlled.
Importance of the slow wave frequency

Electrical slow waves set the maximal rate of contractions in the intestine by restricting action potential generation and force of contractions to the plateau phases (see figure 2). The slow wave frequency, in addition, plays another important physiological role in setting the direction of propagating intestinal contractions. As discussed above, the slow wave frequency exhibits an intrinsic frequency gradient with the highest and lowest intrinsic frequencies being respectively recorded in most oral and aboral segments of the small intestine. This is particularly observed when small pieces of tissue are taken out of the organ and the slow wave frequencies recorded with intracellular or extracellular electrodes. For example, in the murine small intestine, the intrinsic in vitro slow wave frequencies recorded from the very small pieces with microelectrodes at sites 0.5 and 6 cm from the pylorus are \( \sim 54 \) and \( 35 \) cpm, respectively (Der-Silaphet et al. 1998) (figure 3). In comparison, in an intact segment, the in vivo recorded frequency is \( \sim 47 \) cpm at both sites. Hence, the in vivo frequencies of the 0.5 and 6 cm sites are respectively lower and higher than the intrinsic frequencies. The primary reason for the change in the frequency of the 6 cm site is entrainment or ability to increase the frequency of the recorded slow wave by extrinsic stimuli (Mills and Taylor, 1971; Specht and Bortoff, 1972; Cheung and Daniel, 1980). In the case of the 6 cm site, the intrinsic slow wave frequency of this site is overtaken by the higher intrinsic frequency of the oral site that electrically propagated through low resistance pathways (i.e. gap junctions), which now sets the frequency of this site. The electric coupling is also responsible for the decrease in the in vivo frequency of the 0.5 cm site below its own intrinsic frequency. Thus, the oral
**Figure 3.** Propagating electrical activities and intrinsic slow wave frequencies in the proximal murine small intestine. Top left panel shows three simultaneous recordings of electrical activity measured with extracellular suction electrodes from the first 6 cm of the small intestine. Recordings were made from flat tissue with the distance between electrodes ~6 mm (top trace, most proximal to pylorus). Spontaneous slow waves with superimposed spikes are observed, synchronized and apparently propagating in the aboral direction. The slow wave frequency at the three sites is identical at 44 cpm in this example. Boxed area at the bottom shows an enlarged view of the electrical activity. Top right panel represents intrinsic slow wave frequencies recorded with microelectrodes from sites taken from the proximal small intestine at 0.5 and 6 cm from the pylorus. In each case, recordings were made from 3x3 mm pieces of tissue. The 0.5 cm site (top trace) shows the slow wave frequency at 48 cpm, whereas the 6 cm site (bottom trace) at 35 cpm. Adapted from Der-Silaphet et al. (1998).
sites with higher intrinsic slow wave frequency pace the aboral pieces with lower intrinsic slow wave frequency (Diamant and Bortoff, 1969; Szurszewski, 1987). Due to electric coupling between smooth muscle cells in the small intestine, a time lag forms. Slow waves are first observed at oral sites (e.g. 0.5 cm site from pylorus) and then latter at aboral sites (e.g. 6 cm site form pylorus) giving an appearance of the slow wave propagation in the aboral direction or, more specifically, in the direction of the decrease of the intrinsic slow wave frequency gradient. Furthermore, since contractions are usually restricted to the plateaus of the slow waves, and since slow waves propagate in the aboral direction, an apparent propagation of contractions is also observed in the same direction (Daniel and Sarna, 1978; Huizinga et al. 1997). When intestinal contractions are strong enough, the intestinal contents are pushed in the net aboral direction.

**Interstitial cells of Cajal in the small intestine**

ICC were originally described by Ramon Cajal in 1893 and latter in 1911 (Ramon y Cajal, 1893; 1911). Cajal thought of these cells as ‘primitive neurons’. This view of ICC was, however, not shared by Taxis (1953; 1964), who concluded that these cells were neither neurons nor Schwann cells. Most recent studies, reviewed elsewhere (Malysz and Huizinga, 1998), have unquestionably shown that ICC are non-neural in origin and are derived from mesenchyme of the gut.

Until recently, the conclusive differentiation of ICC from other cell types relied on strict electron microscopic criteria. These include both myoid or smooth muscle-like cytological features (presence of the basal lamina, surface caveolae, membrane associated
dense bodies, abundant smooth endoplasmic cisterna, numerous mitochondria, and well developed and organized filament system) and fibroblast-like features (well developed secretary apparatus, in particular frequent dilated cisternae of granular endoplasmic reticulum) (Rumessen and Thuneberg, 1996; Thuneberg, 1989). As shown in this thesis (see Chapters Three through Six) and in Torihashi et al. (1995), ICC can be labeled with anti-kit antibodies or Kit cRNA probes. Thus, we now have a selective way of identifying ICC. Using this marker, the absence or abnormality of ICC have been recently demonstrated in various gastrointestinal disorders including Hirschprung’s disease (Vanderwinden et al. 1996a), infantile hypertrophic pyloric stenosis (Vanderwinden et al. 1996b), chronic idiopathic intestinal pseudo-obstruction (Isozaki et al. 1997) and anorectal anomalies (Kenny et al. 1998b). As a result, the possibility was raised that ICC may play a role in pathogenesis of these gastrointestinal disorders.

In the small intestine, ICC are found as networks of cells associated with two neuronal plexuses: with the deep muscular plexus (in the inner circular muscle layer) and with the myenteric (or Auerbach’s) plexus (between the circular and longitudinal muscle layers). In other organs of the GI tract (e.g. stomach), ICC are also interspersed between the smooth muscle cells of the external muscle layers (Ward et al. 1998; Burns et al. 1996). However, the murine small intestine, the animal model studied in this thesis, lacks ICC of the interspersed type (Burns et al. 1996). The heterogeneous distribution of ICC suggests that they play a number of physiological roles. They have been proposed to function as pacemaker cells responsible for the generation of the slow wave, to facilitate active propagation of electrical events, to mediate neurotransmission, and to function as
mechanoreceptors (Thuneberg, 1982; Sanders, 1996; Huizinga et al. 1997). Of the proposed functions, the evidence is strongest for the pacemaker function and is outlined below.

**ICC as putative pacemaker cells in the small intestine**

The first line of evidence for the function of ICC as pacemaker cells was provided by dissection studies conducted in the 1970's and 1980's. These studies essentially showed that the boundary region between the longitudinal and circular muscle layers in the small intestine is essential for the electrical slow wave activity. As pointed out by Faussone-Pellegrini et al. (1977) and Thuneberg (1982), this area corresponds to the location of ICC, thus, providing indirect support for the pacemaker role of ICC. Several investigators chose to study the feline small intestine in dissection studies (Connor et al. 1974, 1977, 1979; Suzuki et al. 1986). Connor et al. (1974) showed that the circular muscle layer pulled away from the longitudinal muscle layer did not generate slow waves, and they also reported that longitudinal muscle layer removed from the circular muscle layer exhibited slow waves but only in specific areas. Further studies on the feline small intestine revealed the presence of spontaneously actively generating slow waves in only 10-20% of preparations of the longitudinal muscle (from which the circular muscle layer was removed) (Connor et al. 1977). Suzuki et al. (1986) also confirmed these observation in the feline small intestine; they also identified the highest amplitude of the slow waves occurring at the boundary region between the circular and longitudinal muscle layers. Similarly, Hara et al. (1986) illustrated that slow waves were generated by
non-neural cells located between the longitudinal and circular muscle layers in the small intestines of several mammalian species (dog, cat, rabbit, opossum and human). Importantly, they observed a relationship between the slow wave amplitude and the distance away from the myenteric area in the circular muscle layer.

More specific evidence for the role of ICC was provided with the use of methylene blue. This agent under certain conditions selectively labels myenteric ICC in the intestine (Thuneberg, 1982). In the murine (Thuneberg et al. 1983) and feline small intestines (Suzuki et al. 1986), treatment of these tissues with methylene blue led to the loss of ability to record slow waves from the stained preparations. Nonetheless, the possibility that methylene blue abolition of the slow wave was caused by some other effect, not related to the lesion of ICC, still remained.

As discussed in Chapters Three - Six of this thesis, spontaneously genetic mutant mice that have very few or no myenteric ICC (i.e. $W^\prime/W^\prime$, $Sl/Sl^d$, and $W^{ad}/W^{ad}$ mice, for detailed descriptions of the mutations see pages 25-26) exhibited no slow wave activity in the murine small intestine. Since no other cell type appeared to be grossly affected by the mutations, it was concluded that the absence of the slow wave was most likely due to the loss of ICC. Furthermore, recently Thomsen et al. (1998) isolated ICC from the murine small intestine. The isolated ICC exhibited regular inward current oscillations under voltage clamp conditions. The current oscillations had characteristics similar to the slow waves recorded in tissue; in particular, they displayed an insensitivity to L-type Ca$^{2+}$ channel blockade. Hence, studies on the mutant mice and on isolated ICC provided
essential pieces of evidence required for the identification of ICC as pacemaker cells in the small intestine.

**Mechanism of the slow wave generation**

The nature of the ionic basis of the slow wave in the small intestine (as well as in the stomach and colon) remains currently unknown. Several hypotheses have been proposed. In 1965, Daniel (1965) observed that slow waves recorded from the canine small intestine were reduced or blocked by metabolic inhibitors, by decreased extracellular Na\(^+\) (by \(\sim 90\%\)) and with ouabain, an inhibitor of the plasmalemmal Na\(^+\)-K\(^+\) ATPase. The sensitivity of slow waves to ouabain was later confirmed for the feline small intestinal slow waves (Kobayashi et al. 1967). Based on these observations, Daniel proposed the oscillating electrogenic Na\(^+\)-K\(^+\) pump hypothesis as the mechanism responsible for the slow wave generation (Daniel, 1965). According to this view, the upstroke or depolarization phase of the slow wave resulted from the turning off and the repolarizing phase from the turning on of the electrogenic Na\(^+\)-K\(^+\) ATPase pump. Consistent with this hypothesis were observations illustrating the maximal efflux of Na\(^+\) ions occurring during the repolarization phase of the slow wave (Job, 1969), the insensitivity of the triggering mechanism to voltage changes (Connor et al. 1974), and the inability to record conductance changes during the slow wave cycle in the feline small intestine (Connor et al. 1974). The slow wave sensitivities to temperature changes (El-Sharkawy and Daniel, 1975a; Dahms et al. 1987) and to hypoxia (Daniel, 1965) as well as the finding showing that oscillations in intracellular levels of NAD/NADH
corresponded to the slow wave oscillations (Connor et al. 1976) were also consistent with metabolic regulation of the slow wave although not necessarily supporting the oscillating pump hypothesis. In 1975, El-Sharkawy and Daniel re-evaluated the oscillating electrogenic Na\(^+\)-K\(^+\) pump hypothesis, and they concluded that the pump plays a minor role in the generation of the slow wave (El-Sharkawy and Daniel, 1975a; 1975b; 1975c). Their argument was based on the following observations. First, in the presence of ouabain or in the Na\(^+\) -free solution, both conditions expected to inhibit the Na\(^+\)-K\(^+\) ATPase, the resting membrane potential did not depolarize to the level reaching the plateau phase of the slow wave as predicted by the pump hypothesis (Liu et al. 1969; El-Sharkawy and Daniel, 1975a). The membrane potential was, however, significantly depolarized by ouabain and the Na\(^+\) -free solution. Second, the temperature sensitivity of the rabbit small intestinal slow waves (amplitude and resting membrane potential) was much less than would be predicted if the effects of the temperature on the slow wave were mediated through an effect on the pump (El-Sharkawy and Daniel, 1975a). Third, the contribution of the electrogenic pump to the membrane potential was calculated to be much less than would be required to account for the full slow wave amplitude (El-Sharkawy and Daniel, 1975b). Fourth, under certain experimental conditions, a reciprocal relationship was found between the pump activity and the slow wave amplitude (El-Sharkawy and Daniel, 1975b). Fifth, in contrast to a previous study (Connor et al. 1974), changes in membrane conductance were identified during the slow wave oscillation (Mills and Taylor, 1971). Sixth, the Na\(^+\) influx was maximal during the upstroke phase of the slow wave (Job, 1969). To account for the last two observations, Job (1971)
suggested that accumulation of intracellular ATP through synthesis by mitochondria
beyond a critical level activated Na⁺ influx, hence, generating the slow wave. The
repolarization phase of the slow wave, according to Job, was due to the activity of the
pump that activated in the response to accumulation of intracellular Na⁺. Since Job’s
hypothesis required further experimental support, which was not provided, this view was
not generally accepted. An alternative view, the conductance change hypothesis, for the
generation of intestinal slow waves was proposed by El-Sharkawy and Daniel (1975c).
They suggested that the upstroke of the slow wave was caused by an increase in Na⁺
conductance and the slow wave plateau by an increase in Cl⁻ conductance. Their evidence
relied in large part on studies showing the effects of reduced extracellular Na⁺ and Cl⁻ on
the rabbit small intestinal slow waves (El-Sharkawy and Daniel, 1975c). In contrast,
another report, while confirming the effect of the low extracellular Na⁺, failed to find any
effect of the low extracellular Cl⁻ on the feline slow waves (Liu et al. 1969). The El-
Sharkawy and Daniel’s conductance model, however, does not explain the sensitivity of
the slow wave (amplitude and frequency) to extracellular Ca²⁺. Several investigators have
shown that reduction of the extracellular Ca²⁺ decreased the slow wave amplitude and
frequency in the small intestines of cats (Tamai and Prosser, 1966; Dahms et al. 1987),
rabbits (El-Sharkawy and Daniel, 1975c), dogs (Cayabyab et al. 1996), and mice
(Chapter Seven of this thesis). To their defense, El-Sharkawy and Daniel (1975c)
suggested that the inward Na⁺ conductance may be dependent on the presence of
extracellular Ca²⁺ or the triggering mechanism of the slow wave may be Ca²⁺ dependent.
Further support for the involvement of voltage dependent conductance changes in the
generation of the small intestinal slow waves was provided by observations showing that the natural slow wave frequency could be entrained or driven to a higher value by passing short pulses extracellularly (Mills and Taylor, 1971; Cheung and Daniel, 1980; Specht and Bortoff, 1972) and that the slow wave frequency and amplitude could be altered by changing the membrane potential by passing a constant current (Connor et al. 1974). The effect of the membrane potential on the slow wave was, however, relatively weak. In the study by Conner et al. (1974), a 10 mV hyperpolarization increased the slow wave amplitude from ~8 to ~11 mV and decreased the slow wave frequency from ~14 to ~7 cpm in the feline small intestine. Furthermore, this study also illustrated that when the membrane potential was clamped at the resting membrane potential, current traces appeared at the same frequency as the slow waves. The latter two findings are difficult to explain by a voltage activated conductance change being responsible for the initiation of the slow wave. It is also relevant to the point, in the context of this discussion, that several investigators (Bortoff, 1976; El-Sharkawy and Daniel, 1975c) doubt whether it is possible to clamp the membrane potential of a multicellular three-dimensional preparation such as the feline small intestinal preparation utilized by Connor and co-workers.

In summary, studies investigating the ionic mechanism of the slow wave initiation and generation have identified the involvement of the voltage dependent conductance change and a metabolic process, other than that mediated by the $\text{Na}^+\text{-K}^+$ ATPase pump, in the slow wave generation. The relationship between these two processes remains unclear. A possible connection could be that some unknown metabolic event triggers the
slow wave by activating an ionic conductance mediated by Na⁺, by Ca²⁺, or by both. This view was previously considered by Conner et al. (1974), but it was not supported or investigated in their future studies. A similar hypothesis was also formulated to account for the guinea pig antral slow waves. In this organ, some active ion transport system was proposed to be responsible for the generation of the first slow wave component that was followed by the second, potential dependent slow wave component (Tomita, 1981).

**Calcium release from intracellular stores**

Cytoplasmic free Ca²⁺ concentrations play an important role in the regulation of a variety of cellular functions depending on the cell type. In muscle cells including the smooth muscle types, initiation of contractile activity depends directly on Ca²⁺. Intracellular Ca²⁺ concentrations are regulated by the influx of the ion through plasmalemmal channels (e.g. L- or T-type Ca²⁺ channels), carrier transport mechanisms (e.g. Na⁺/Ca²⁺ exchanger), or by release of Ca²⁺ from the intracellular pools or stores (Missiaen et al. 1992; Karaki et al. 1997). The last route includes two separate sarcoplasmic reticulumum (SR) release mechanisms involving either the inositol (1,4,5) triphosphate (IP₃) receptor or the ryanodine receptor, and they are known as IP₃ induced and Ca²⁺ induced Ca²⁺ release mechanisms, respectively (lino, 1990).

The IP₃ induced Ca²⁺ release (IICR) as the name specifies is activated by IP₃, an intracellular second messenger. IP₃ is generated together with DAG (diacylglycerol) from a breakdown of phosphatidylinositol (4,5) bisphosphate (PIP₂) catalyzed by a class of phospholipases, specifically phosphatidylinositol specific phospholipases C (PLC)
(Berridge, 1993a; Berridge, 1993b). These phospholipases form a broader superfamily of PLC. In mammalian cells at least three distinct families of PLCs are recognized: PLC-β, PLC-γ, and PLC-δ with at least 4, 2, and 4 different members in each family, respectively. In contrast to PLCs-γ and PLCs-δ, which are mainly localized in the cytosol, the PLC-β family is found localized within the plasmalemma associated with G-proteins (James and Downes, 1997; Exton, 1996). Functional diversity is created by different receptors to which agonists or neurotransmitters bind being coupled to the distinct G proteins and PLCs β. For example, in the iris sphincter smooth muscle, muscarinic receptors are associated with Gq proteins and with PLC β1 (Zhou et al. 1994). Both products of the PLC catalyzed breakdown of PIP2, IP3 and DAG function as intracellular messengers, but only the former has been shown to release Ca2+ from intracellular pools. The release mechanism is quantal (Parys et al. 1996), does not show any voltage dependency (Ehrlich and Watras, 1988), is blocked by heparin, and activated or inhibited by ATP or Ca2+ in a concentration dependent manner (Mayrleitner et al. 1991; Maeda et al. 1991; Iino, 1990). Concentrations of Ca2+ ranging from 0 to 300 nM were found to stimulate the release and those above 300 nM to inhibit the IIICR. The activation of the receptor by ATP and Ca2+ requires the presence of IP3, and in its absence, the stimulation with these factors is not possible (Conley, 1996). Other known pharmacological modulators of the IP3 receptor and of IP3 induced Ca2+ release are caffeine, thimerosal, and 2-aminoethoxydiphenyl borate that respectively block (at 5-20 mM) (Parker and Ivorra, 1991), sensitize (Joseph et al. 1995), and stimulate (above 90 μM) the Ca2+ release (Maruyama et al. 1997). In addition, the luminal Ca2+ content
within the intracellular stores also appears to control the release since more IP$_3$ was needed to deplete the stores with a lower Ca$^{2+}$ content than with more filled stores (Missiaen et al. 1992). Cloning and purification studies identified at least three different isoforms of IP$_3$ receptors (IP$_3$I to IP$_3$-III), each encoded by a separate gene (Conley, 1996; Wilcox et al. 1998). The relative functional differences among the types have not yet been fully characterized, but differences in affinity for IP$_3$ binding have been reported. When IP$_3$ receptors were expressed in bacteria, the affinity of IP$_3$ for the IP$_3$-III subtype was 10-fold less than that for the IP$_3$I subtype (Newton et al. 1994).

The Ca$^{2+}$ induced Ca$^{2+}$ release (CICR) involves a different mechanism utilizing ryanodine receptors (RyRs). These receptors are named after their high affinity or selectivity for ryanodine, a plant alkaloid. Pharmacologically, this Ca$^{2+}$ release mechanism can be stimulated with Ca$^{2+}$, ryanodine, ATP, and caffeine, whereas inhibition has been reported with Mg$^{2+}$, procaine, dantrolene, and ruthenium red (Coronado et al. 1994). The effect of ryanodine on the Ca$^{2+}$ release is complex and depends on the concentration of the plant alkaloid. In liver microsomes, concentrations in the range of 0.01-10 μM caused stimulation of the release, while higher concentrations (10-300 μM) produced inhibition (Bazotte et al. 1991). Similar observations were reported in studies on the skeletal and cardiac SR (Gilchrist et al. 1992; Humerickhouse et al. 1993; Lattanzio et al. 1987). The only two endogenous activators identified so far for the CICR are Ca$^{2+}$ and cyclic ADP ribose (cADPR) (McPherson and Campbell, 1993; Lee, 1997). The latter modulator may be of physiological importance. It is a metabolite of NAD$^+$ whose formation is catalyzed by ADP-ribosyl cyclases. These
enzymes are not specific and can act on many different analogs of NAD producing corresponding analogs of cADPR (Walseth and Lee, 1993). ADP-ribosyl cyclases are also potential targets for regulation by stimuli or factors including cGMP, NO, and retinoic acid (Lee, 1997); hence, they may play a role in intracellular signaling.

Molecular biological methods have identified three separate mammalian ryanodine receptor (RyR) genes, which are designed as ryr-1, ryr-2, and ryr-3 (Coronado et al. 1994; Sorrentino and Volpe, 1993). They respectively encode RyR-1, RyR-2, and RyR-3 receptor proteins. Due to the predominant expression of each subtype in a given tissue, RyR-1 are also known as the skeletal ryanodine receptors, RyR-2 as the cardiac ryanodine receptors, and RyR-3 as the brain ryanodine receptors. The distribution of each type, however, is much broader. For example, RyR-2 are also found in brain, and RyR-2 and RyR-3 in smooth muscle cells (Coronado et al. 1994; Sorrentino and Volpe, 1993).

In various muscle systems, IICR and CICR mechanisms have been linked to coupling processes involved in the control of muscle contractility (Berridge, 1993b; Coburn and Baron, 1990; Delbono et al. 1997). The IICR plays a role in the pharmacokinetic or pharmacomechanical coupling through which external agonists or neurotransmitters generate IP$_3$ by stimulating receptors linked to G-proteins and PLC. This intracellular messenger, by releasing Ca$^{2+}$ from the intracellular stores, initiates the contractile activity independently of the changes in the membrane potential and of the influx of Ca$^{2+}$ through the plasmalemma. The latter two processes play a critical role in the activation of CICR. In skeletal muscles, the plasmalemmal depolarization is transmitted to ryanodine receptors via physical interactions resulting in Ca$^{2+}$ release, and
in cardiac cells influx of \( \text{Ca}^{2+} \) in response to the depolarization activates CICR (McPherson and Campbell, 1993; Franzini-Armstrong and Protasi, 1997). In smooth muscle cells including those of the intestine, the role of CICR during the excitation contraction coupling remains unclear.

**Kit tyrosine kinase receptor-steel factor interaction**

Membrane bound kit receptor contains an intrinsic tyrosine kinase activity, which is activated when the receptor is stimulated with its ligand, the steel factor (Besmer et al. 1993; Huang et al. 1990). The interaction between the two proteins has been shown to be essential for migration, survival, and proliferation of melanocytes, gametocytes, and hematocytes (Bernstein et al. 1990; Nocka et al. 1989). As a result, \( W/W^c \) and \( S1/S1^d \) mice, which respectively have mutations affecting the structure of the kit receptor and its ligand (steel factor), exhibit an identical phenotypic pattern. They are white, sterile, lack tissue mast cells, and have macrocytic anemia due to abnormalities in these three processes. In contrast, wild type animals are black, fertile, have unaffected mast cells, and without anemia (Nocka et al. 1990; Silvers, 1979; Nocka et al. 1989).

In 1992, Maeda et al. (1992) reported that injection of anti c-kit antibodies into newborn mice resulted in severe abnormalities of the gut movements as well as in a reduction of c-kit positive cells in the muscularis externa, particularly at the location in the proximity of the myenteric plexus, but neural bodies and processes were not affected. Based on these and other observations, Maeda et al. (1992) proposed that "c-kit plays a crucial role for development of a component of the pacemaker system that is required for
the generation of autonomic gut motility". Furthermore, they identified that the \( W/W^v \) murine small intestinal musculature, similarly to the c-kit injected murine muscle, lacked normal phasic contractile activity (Maeda et al. 1992). Abnormal motility patterns in \( W/W^v \) mice were also previously suggested by a prolonged rate of removal of worms *Trichinella spiralis* (Kamiya et al. 1985) and *Nippostrongylus brasiliensis* (Crowle, 1983) from the infected intestine as compared to controls.

Recently, two other \( W \) mutations (\( W^{bd} \) and \( W^{s7} \)), both affecting the transcriptional initiation of the \( W/kit \) gene, have been characterized. The \( W^{bd} \) mutation involves a 2.8 Mb genomic inversion of a sequence upstream of the \( W/kit \) gene, while the \( W^{s7} \) mutation has a 80 kb deletion also upstream of the \( W/kit \) gene (Klüppel et al. 1997). Although in \( W^{bd}/W^{bd} \) and \( W^{s7}/W^{s7} \) mice melanocyte and mast cells function similarly to \( W/W^v \) mice is abnormal, gametocytes develop normally, and the mice are fertile (Klüppel et al. 1997). Effects of the \( W^{bd} \) and \( W^{s7} \) mutations on the ICC and the slow wave activity are described in Chapter Six of this thesis.
Chapter Two

Objectives, Methodology and Thesis Outline

Objectives

Two broad issues, the cellular origin and the mechanisms of the generation and regulation of the electrical slow wave, remained unresolved at the time when studies discussed in this thesis were undertaken. Both became a central theme of the current thesis. As outlined above and discussed in detail elsewhere (Huizinga et al. 1997; Sanders, 1996), evidence over the last two decades points to ICC associated with the myenteric plexus in the small intestine being responsible for the generation of the slow wave. To provide evidence for the proposed cellular origin of the slow wave, experiments on spontaneously genetic mutant mice ($W/W^r$, $Sl/S^{pr}$, $W^{bd}/W^{bd}$) have been performed.

In Chapters Three and Four, the morphological and electrophysiological features of the small intestines of $W/W^r$ and control mice were examined. Since the mutant mice as reported in Chapters Three and Four have few or no myenteric ICC, they offered a unique opportunity to examine the proposed role of ICC as pacemaker cells responsible for the generation of the slow wave. Since the standard microelectrode technique is the most appropriate method to record the electrical activities of the musculature, I utilized this technique to study the electrical behavior of the small intestinal musculature of $W/W^r$ and control mice. The expected finding was that these mutant mice lacked slow waves; however, the exact nature of the electrical activity in the absence of the slow wave could
not be predicted. Thus, careful experiments on the mutant and control mice were carried out including characterization of the responses to pharmacological agents including L-type Ca^{2+} channel and K^{+} channel blockers.

To confirm that the effects on the ICC and the slow wave activity in the W/\bar{W} mice were attributed to the abnormality in the steel factor - kit receptor interaction, additional experiments were carried out on other spontaneously genetic mutant mice. In Chapter Five, the structural and electrophysiological features of the Sl/Sld murine small intestine were examined. In Chapter Six, the analyses were performed on W^{bd}/W^{bd} and W^{57}/W^{57} mutants. For each mutant mouse and genetic control strain, I carried out electrophysiological experiments utilizing the standard microelectrode technique. Experiments on various mouse strains were needed to support the hypothesis that steel factor - kit receptor interaction sometime during the postnatal development is required for the presence of the network of myenteric ICC and the slow wave pacemaker activity in adult animals.

The mechanisms of the slow wave generation and regulation remain currently largely unresolved. One of the major objectives of my research was to provide an insight into the ionic mechanisms of the slow wave initiation focusing on roles of Na^{+}, Ca^{2+}, and Cl^- . This was a primary goal of Chapter Seven and in part of Chapter Nine. In these studies, I applied the standard microelectrode technique to record slow waves from CD1 mice. Effects of reduction of extracellular Na^{+}, Ca^{2+}, and Cl^- as well as Na^{+} channel, Ca^{2+} channel, Cl^- channel, and nonselective cation channel blockers were examined on the
slow wave. The objective was to identify ions and channels that are most important for the generation of the slow wave and to propose a model for the slow wave formation.

In Chapters Eight and Nine, the roles of the intracellular Ca\(^{2+}\) and intracellular Ca\(^{2+}\) release mechanisms (CICR and IICR) were examined in the regulation of the slow wave and membrane potential in the small intestinal musculature of CD1 mice. By determining the effects of blockers or activators of the Ca\(^{2+}\) release mechanisms on the electrical activity as recorded with the standard microelectrode technique, it was hoped that a critical regulatory pathway will be identified. These studies were important since little is known about mechanisms of the slow wave regulation and no previous study is available addressing the roles of CICR and IICR as examined in this thesis.

**Choice of animal model**

The choice of the murine small intestine for electrophysiological investigation offers several advantages including the extensive available literature on the morphological characterization of the small intestine of this species (Thuneberg, 1989; Young et al. 1996; Torihashi et al. 1997; Faussone-Pellegrini, 1984; Faussone-Pellegrini and Cortesini, 1984), the availability of spontaneously genetic knock out mutant mouse strains (Nocka et al. 1990; Silvers, 1979; Nocka et al. 1989; Klüppel et al. 1997). For all of the mutant strains used in this thesis, previous studies clearly established the genetic basis of mutations. The narrow thickness of the muscularis externa of this species as compared to other available models offers an advantage in minimization of the variability associated with thick tissues. Furthermore, the murine small intestine is
currently being used in Dr. Huizinga's laboratory and also at other locations to isolate smooth muscle cells and ICC. Consequently, significant insights can be made possible by combining data from isolated cells and tissue work.

**Methodology and Thesis Outline**

Chapters Three through Nine are in formats of manuscripts, each with its own list of references. A general introduction and discussion are respectively included in Chapters One and Ten. The list of references for these two chapters as well as for Chapter Two is included on pages 203 - 214. With the exception of Chapters Seven through Nine where the presented data relied on the standard microelectrode technique, a variety of techniques involving different investigators were utilized including light and electron microscopy, immunohistochemistry, *in situ* mRNA hybridization, and a standard microelectrode technique. Each technique and contribution of each investigator is in detail described in each research chapter. The descriptions of methods are self-explanatory and are not repeated in this chapter.
Chapter Three


\textbf{Methods and contributions}

John Malysz was involved in all aspects of the development of the project from very beginning and was the major force behind development of the technique for microelectrode cellular recording in the murine small intestine. Using this technique, John Malysz performed all electrophysiological and carried out all analysis of electrophysiological data in this manuscript; he also took part in writing this paper. Dr. Lars Thuneberg carried out all light and electron microscopic studies. Dr. Hanne Mikkelsen performed the anti kit receptor immunohistochemistry and Dr. Micheal Klüppel (in Dr. Alan Bernstein’s laboratory) the \textit{in situ Kit} mRNA hybridization studies. Dr. Jan D. Huizinga initiated the studies, was involved in study design and writing, and coordinated all projects.
W/κit gene required for interstitial cells of Cajal and for intestinal pacemaker activity

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The pacemaker activity in the mammalian gut is responsible for generating anally propagating phasic contractions. The cellular basis for this intrinsic activity is unknown. The smooth muscle cells of the external muscle layers and the innervated cellular network of interstitial cells of Cajal, which is closely associated with the external muscle layers of the mammalian gut, have both been proposed to stimulate pacemaker activity.1,2 The interstitial cells of Cajal were identified in the last century but their developmental origin and function have remained unclear. Here we show that the interstitial cells of Cajal express the Kit receptor tyrosine kinase. Furthermore, mice with mutations in the dominant white spotting (W) locus, which have cellular defects in haematopoiesis, melanogenesis and gametogenesis as a result of mutations in the Kit gene2, also lack the network of intestinal cells of Cajal associated with Auerbach’s nerve plexus and intestinal pacemaker activity.

Injection of antibodies directed against the extracellular domain of the Kit receptor tyrosine kinase into newborn mice leads to changes in in utero contraction patterns in the small intestine and absence of Kit messenger RNA in the myenteric plexus area. This observation prompted us to investigate whether the Kit receptor might play a role as a signalling molecule required for the development of the interstitial cells of Cajal (ICC) and therefore be essential for intestinal pacemaker activity. To determine whether mutations at the murine W/κit locus might affect normal development of ICC, we first examined the morphology of the intestines of mutant W/ W' mice and their control littermates. Control mice (albino, +/+ and W'/+) had normal ICC networks in the small intestine, visualized by selective uptake of methylene blue (Fig. 1a) and confirmed by electron microscopy (Fig. 2a, b). By contrast, the network of ICC in the myenteric plexus region was absent in W/ W' mice (Fig. 1a), again confirmed by electron microscopy (Fig. 2c, d). In the mutants, only scattered methylene-blue-positive cells which might be genuine ICC were observed (Fig. 1b). Using electron microscopy, we counted 51 ICC along a length of 1.109 cross-sectioned muscle cells bordering the myenteric plexus in W/ W' control mice; by contrast, only 7 ICC were observed along a length of 3.059 muscle cells in W/ W' mice. Quantitatively, 70% of muscle cells bordering the Auerbach’s plexus contacted ICC in control mice, compared with only 1.5% in W/ W' mice.

We also observed the direct apposition of large stretches of the circular and longitudinal smooth muscle layers in W/ W' mice in the absence of the intermediate network of ICC found in normal animals (Fig. 2c). Light and electron microscopic analysis of duodenal muscularis revealed no differences in the density, morphology or ultrastructural features of enteric neurons or glial cells between +/+ and W'W' mutant mice. indicating

FIG. 1. Histochemical analysis of the small intestine in wild-type and W mutant mice. a, b, Double staining of interstitial cells of Cajal associated with Auerbach’s plexus (ICC) (shown as the stained cellular network using vital staining with methylene blue) and macrophages (fluorescence; ingestion of fluorescein isothiocyanate FITC-labeled dextran). Whole mounts of the isolated duodenal mucosa externa of a control albino mouse (a) and W/ W' mouse (b); a is representative of the normal staining pattern of both cell types in albino mice as well as W/ W' mice. Normally, ICC together with macrophages form a partial sheath around ganglia and primary fascicles of Auerbach’s plexus (AP) but in b, only macrophages are present in normal numbers and normally organized. c–e, Sectioned jejunal tissue, c, 1 μm section of Epon-embedded tissue, processed with preservation of methylene blue, no post-staining. ICC are seen as the blue, broken line between the two layers of the muscularis externa (between dashed lines). Arrowheads point to unstained, partially enveloped elements of Auerbach’s plexus (compare with a). d, e, Frozen 8-μm sections, showing Kit immunoreactivity in ICC in a W/ W' mouse and its absence in the W/ W' mouse (e). Magnification, x225 (a); x320 (b–e); scale bar, 25 μm.

METHODS. Methylene blue was preserved through dehydration and embedding by precipitation as a hexachloroplatinate15. Immunocytochemistry was performed on unfixed frozen sections of small intestines from W/ W' mice and their controls. Both the PAP and the biotin–strepavidin Texas-red methods were used15.
that defects in the Kit signalling pathway do not affect neuronal or glial cells in the Auerbach's plexus region.

These data indicate that a functional Kit receptor is required for the development of ICC in the Auerbach's plexus region of the small intestine. To determine whether the ICC deficiency in W"/W" mutant mice was a cell-autonomous defect, we analysed Kit RNA and protein expression in W"/W" mutant mice and control animals. We used a polyclonal antibody directed against the intracellular domain of the Kit receptor tyrosine kinase to localize the protein. In wild type and W"/+ high levels of Kit expression were observed between the longitudinal and the circular muscle layers at the level of Auerbach's plexus (Fig. 1d). The stained cells surrounded the ganglia and their distribution in the Auerbach's plexus area was identical to that of the methylene-blue-stained ICC (Fig. 1e). By contrast, no Kit immunoreactivity was found in or between the muscle layers of W"/W" mice (Fig. 1e).

We next performed whole-mount RNA in situ experiments on the ileum of wild-type and W"/W" mice. The ileum of +/+ mice contained Kit-positive cells whose organization was identical to that of ICC, as revealed by methylene-blue staining (Fig. 3a). These cells were localized between the longitudinal and circular

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**Fig. 2.** EM analysis of the small intestine of wild-type and W"/W" mutant mice. a–d. Electron micrographs of the jejunal myenteric plexus area in an albino mouse (a), a W"/+ mouse (b), and a W"/W" mouse (c, d). a. Normal appearance of ICC in the Auerbach's plexus region (i), tightly associated with nerve fascicles (N) of Auerbach's plexus, between the longitudinal (LM) and circular (CM) muscle layers. An abundance of mitochondria (arrows) and caveolae are distinctive ultrastructural features of ICC. b. Pretaining with methylene blue assists the differentiation of ICC (i) with increased granularity and electron-density of nuclei and ribosomal areas) from fibroblasts (F), macrophages (M, macrophage process) and pericytes (C, capillary). c–d. The overall organization of the muscle layers seemed normal. Although cell types other than ICC were counted in normal numbers in W"/W", one result of the absence of ICC was a strong increase in the extension of areas of direct apposition of muscle cells of LM and CM (c). Sheath cells around nerves were either absent (d) or small tertiary nerve between the muscle layers) or had fibroblast ultrastructure.

Tissues were fixed and processed for electron microscopy (Philips 300 microscope) by routine methods. Scale bars: 2 μm (a–c); 1 μm (d).

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**Fig. 3.** Whole-mount RNA in situ analysis of Kit expression in the small intestine of wild-type and W"/W" mutant mice. a. A network of Kit-expressing cells is present in the isolated external muscle layers in +/+ mice, identical in appearance to the ICC network (compare Fig. 1a). b. Kit-positive cells were absent in similar whole mounts of W"/W" mice. Cross-sectioning of the whole-mount tissues localized the Kit-positive cells in +/+ mice between the longitudinal and circular muscle layer (c; compare Fig. 1c at the same magnification) and confirmed their absence in W"/W" mice (d). Scale bars: 50 μm (a, b); 25 μm (c, d).

**METHODS.** Whole-mount RNA in situ hybridization was performed according to the method of D. Henrique and D. Ish-Horowicz (personal communication), with the following modifications. Protease K digestion was for 30 min at a concentration of 20 μg ml⁻¹. The hybridization mix contained 5 × SSC, 50% formamide, 5 mM EDTA, 50 μg ml⁻¹ yeast tRNA, 0.2% Tween-20, 0.5% CHAPS and 100 μg ml⁻¹ heparin, pH 6.5 with citric acid. After in situ hybridization, tissues were postfixed in 4% paraformaldehyde before being embedding and sectioning. The Kit cDNA probe has been described.
muscle layers in an identical position to the ICC (Fig. 3c). By contrast, no Kit−positive cells were observed in the muscle layers of W/W− mice (Fig. 3d).

To determine whether these morphological and cellular differences between +/+ and W mutant mice were associated with functional anomalies, we measured the electrical activity of the small intestinal muscle layers. Normal mice displayed slow-wave-type action potentials with an amplitude of 21.5 ± 5.9 mV, a frequency of 32.4 ± 1.0 cycles per minute (range 30–36.6 c.p.m.) and a resting membrane potential of −60.0 ± 3.0 mV (Fig. 4; n = 9). By contrast, the lack of W/W− mice failed to display any slow-wave-type action potentials (Fig. 4). The membrane potential was −44.8 ± 1.3 mV (n = 14) and at irregular frequency fast spike-like action potentials arose from it, singly or in groups of 2–6. Their amplitude was 16.4 ± 2.1 mV, the frequency ranged from 4 to 20 c.p.m., and was irregular within one preparation. In 4 out of 14 W/W− mice, no spontaneous action potentials were observed. The slow-wave component or pacemaker activity of gut smooth muscle is insensitive to L-type calcium channel blockers. In the presence of the blockers nifedipine or D600, the slow-wave component of the action potentials in +/+ mice remained unaltered, whereas the electrical activity of W/W− mice was completely abolished (n = 14; Fig. 4). These data demonstrate that mutations at the murine W locus lead to the absence of the ICC network in the Auerbach’s plexus region and of pacemaker activity in the small intestine, demonstrating an essential role for ICC in gut pacemaker activity. Because the ICC express the Kit receptor, we conclude that the absence of these cells in W mutant mice reflects a direct role for Kit in ICC development. The phenotype of W− mutant mice was thus expected to resemble that of in vivo haematopoietic germ and melanocyte lineages, even though Kit and its ligand Steel factor are continguously expressed in additional anatomical sites, including the small intestine. Thus, our experiments extend the range of cell types affected by W mutations to include the interstitial cells of Cajal. Mice with mutations in Steel factor (SI/SIl− mutant mice) display abnormalities in the gut similar to those found here in W mutant mice (data not shown).

Our data suggest that the functional gut abnormalities and megacolon observed in individuals with piebaldism, a hypopigmentation disorder that also results from mutation in the KIT proto-oncogene, reflect an identical function of the Kit signaling pathway in the development of the ICC in humans. Mutations in the RET proto-oncogene are also associated with gut abnormalities in humans (Hirschsprung’s disease) and in genetically targeted mutant mice, as the result of a cellular deficit in neural crest-derived enteric neurons. Thus, two members of the receptor tyrosine kinase family, Kit expressed in ICC and RET expressed in neural crest-derived ganglion cells, are both essential for normal gut function in mammals.

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Chapter Four


**Methods and contributions**

John Malysz performed all electrophysiological experiments, did all analysis of electrophysiological data, and was involved in writing the manuscript and in study design. Dr. Lars Thuneberg carried out all light and electron microscopic studies, and Dr. Hanne Mikkelsen performed the anti kit receptor immunohistochemistry. Dr. Jan D. Huizinga initiated the studies, was involved in study design and writing, and coordinated all projects.
Action potential generation in the small intestine of W mutant mice that lack interstitial cells of Cajal

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Malysz, John, Lars Thuneberg, Hanne B. Mikkelensen, and Jan D. Huizinga. Action potential generation in the small intestine of W mutant mice that lack interstitial cells of Cajal. Am. J. Physiol. 271 (Gastrointest. Liver Physiol. 34): G387–G399, 1996.—The small intestine of W/Wmice lacks both the network of interstitial cells of Cajal (ICC), associated with Auerbach’s plexus, and pacemaker activity, i.e., it does not generate slow-wave-type action potentials. The W/Wmice muscle preparations showed a wide variety of electrical activities, ranging from total quiescence to generation of action potentials at regular or irregular frequency with or without periods of quiescence. The action potentials consisted of a slow component with superimposed spikes, preceded by a slowly developing depolarization and followed by a transient hyperpolarization. The action potentials were completely abolished by L-type Ca²⁺ channel blockers. W/Wmice responded to K⁺ channel blockade (0.5 mM Ba²⁺ or 10 mM tetrathylammonium chloride) with effects on amplitude, frequency, rate of rise, and duration of the action potentials. In quiescent tissues from W/Wmice, K⁺ channel blockade evoked the typical spike-like action potentials. Electron microscopy identified few membrane blue-positive cells in the W/Wmice small intestine associated with Auerbach’s plexus as individual ICC. Numbers of resident macrophage-like cells (MLC) and fibroblast-like cells (FLC) were significantly changed. Neither FLC nor MLC were part of a network nor did they form specialized junctions with neighboring cells as ICC do. Hence no cell type had replaced ICC at their normal morphological position associated with Auerbach’s plexus. ICC were present in W/Wmice at the deep muscular plexus in normal organisation and numbers, indicating that they are not dependent on the Kit protein and do not take part in generation of pacemaker activity.

slow waves; pacemaker activity; excitability; W/Wmice; W mutation; fibroblast; macrophage; Kit receptor

STUDIES ON W/Wmice, with an abnormal kit gene, have provided compelling evidence for the hypothesis that interstitial cells of Cajal (ICC) are the pacemaker cells of the gut. The evidence is based on the observations that W/Wmice exhibit abnormal intestinal motility (15, 24), lack the myenteric ICC (16, 43), and lack intestinal pacemaker electrical activity, the slow-wave-type action potentials (16, 43).

The mutation in W/Wmice, occurring in the W (White spotting) locus, affects not only intestinal function but also various aspects of hematopoeisis, melanogenesis, and gametogenesis (8, 36). The W locus has an unusual rate of mutagenesis (37) that produces many independent W mutations (2, 31, 34). Mutations are semidominant and give a range of alleles with various degrees of severity on the affected systems in comparison with wild-type mice that are fertile, have normal black skin pigmentation, and have normal blood parameters. For example, heterozygous W/+ and W/+ are fertile, only mildly anemic, and have a characteristic white forehead blaze and additional areas of depigmentation on the ventral body, tail, and feet. In comparison, homozygous W/W generally die during embryonic development. Compounds heterozygous strains, specifically W/Wmice, are viable but sterile, with mild to severe anemia, entirely white pigmentation, and black eyes (2, 36).

Recently, it was found that the W locus encodes the Kit protein kinase receptor (4, 9, 30, 31). The receptor consists of an extracellular, a transmembrane, and a cytoplasmic domain. The cytoplasmic domain contains the kinase activity and adenosine 5′-triphosphate (ATP) binding sites (7, 31). The W and Walleles encode, respectively, for a product in which the transmembrane region is missing (11) and one in which a single amino acid substitution occurs within the kinase active site (31). Consequently, the W allele encodes for a product with no kinase activity, and the W allele encodes for a product with little kinase activity (8, 31). Recent immunohistochemical methods revealed the Kit protein (16, 43) and Kit mRNA (16) to be present in ICC.

The ligand for the Kit receptor is the steel factor (14, 45), which is abnormal in SI/SI mice (10, 44). When the observation was made that SI/SI mice also lack the myenteric ICC network (41), the hypothesis emerged that the absence of an interaction between the steel factor and the Kit receptor during ICC development is responsible for the virtual absence of ICC in W/Wmice. In support of this view, injection of antibody against the extracellular domain of the Kit receptor kinase in newborn mice blocks the development of the myenteric ICC network and also results in abnormal intestinal motility in these mice (42).

The present study addresses two critical questions. First, in the absence of pacemaker cells, what type of electrical activities do the smooth muscle cells in W/Wmice generate that allow the intestine to move contents analy? Second, what are the morphological consequences of the absence of ICC?

METHODS

Recording of Electrical Activities

Mice of either sex, W/Wmice, +/+ and W/+ (Jackson Laboratories) and CD1 (Charles River Laboratories), were killed by cervical dislocation. The small intestine was then exposed by a midline abdominal incision, and a segment (5–10 cm) of the ileal small intestine was removed with the distal and approximately 1–2 cm away from the cecum. The removed segments
were placed in a dissection dish filled with oxygenated (95% O₂-5% CO₂) Krebs solution. The preparations were opened flat, and the mucosal contents were removed. The mucosa was then carefully removed by sharp dissection. Mucosal strips, 1.5 mm long, were prepared by cutting parallel to the longitudinal muscle bundles, 0.5–0.8 cm wide. The muscle was mounted on Sylgard on a transfer holder and placed in a bath with a 6-ml capacity. The preparation was perfused with Krebs solution at a constant rate (5.0 ml/min) at 36–37°C. Intracellular recordings were made by microelectrodes with a tip resistance of 40–100 MΩ and a tip diameter of ~150 nm. Microelectrodes were prepared from 1.2-mm OD glass capillaries (WPI) and filled with 3 M KCl. A microelectrode was inserted into a microelectrode holder (WPI M700P) that was connected to an electrometer (WPI M-707A), which is a high-impedance probe. The output of the electrometer was displayed on a Gould oscilloscope (1425) and recorded on a Gould ink-writing recorder.

**Data analysis of electrical activities.** Figure 1 shows actual recordings of electrical activities in control (A) and W/W* (B) mice. In control mice, the following parameters of the slow-wave-type action potentials were measured: the resting membrane potential (RMP), slow-wave amplitude (A), the amplitude of the spike component (S), the average rate of rise of the upstroke (from 0 to 20 mV; RR), and the rate of rise of the spike component (RS). The action potentials of W/W* mice were characterized according to the following parameters: RMP, RS, amplitude of the slow component (A), average rate of rise of the upstroke of the slow component (0–5 mV; RR), amplitude of the hyperpolarization (H), and the average rate of rise of depolarization following hyperpolarization (RD). In addition, in control and W/W* mice, other parameters, including frequency of action potentials, duration measured at one-half maximal amplitude of slow wave (controls) or slow component (W/W* mice), frequency of spiking activity, and number of spikes per action potential were analysed.

Data were obtained usually from the same cells, before and in the presence of a drug or after ion or solution substitution.

A difference was considered significant if P < 0.05. Data are expressed as means ± SE. Paired or unpaired Student’s t-tests were employed for comparisons of the electrical activities and cellular structures. Linear regression analysis was performed on data to determine correlation coefficients (r²) between resting membrane potential and action potential frequency for W/W* and control mice.

**Solutions and drugs.** The physiological experiments were performed in prewarmed (36–37°C) Krebs solution and equilibrated with 95% O₂-5% CO₂. The composition of the Krebs solution was (in mM) 120.3 NaCl, 5.9 KCl, 2.5 CaCl₂, 1.2 MgCl₂, 20.0 NaHCO₃, 1.2 NaH₂PO₄, and 11.5 glucose. The pH was 7.30–7.35. In experiments in which the solution was modified to Ca²⁺-free Krebs, all of the ingredients except CaCl₂ were used. In experiments that required verapamil (Sigma), a final concentration in the range of 10⁻⁴ to 5 × 10⁻⁴ M was obtained. BaCl₂, NiCl₂, tetrodotoxin (TTX), and tetraethylammonium chloride (TEA) were obtained from Sigma (St. Louis, MO).

The animals were cared for in accordance with the principles and guidelines of the Canadian Council on Animal Care.

**Immunohistochemistry**

W/W*, W/+ , and albinos (BALB/c) mice were killed by cervical dislocation. Pieces (3 × 3 mm) of unfixed small intestine were used to store whole mounts and frozen sections. Quick-freezing was done in isopentane cooled to −160°C with liquid nitrogen, followed by storage at −80°C. Whole mounts were pinned with serial side up, on Sylgard (Dow Corning) and fixed in 2% formaldehyde and 15% picric acid in phosphate-buffered saline (PBS) for 18 h (29). The pinned pieces were dehydrated in ethanol and xylene and rehydrated, after which the mucosa and submucosa were removed.

The immunohistochemical techniques were described previously (26, 27). Polyclonal rabbit antibodies (16) and monoclonal rat antibodies [immunglobulin (Ig) G₂a; Pharmingen] against, respectively, the intra- and extracellular domain of mouse Kᵢ protein tyrosine kinase receptor were used. To demonstrate glial cells, rabbit antibodies against glial fibrillary acidic protein (GFAP; Z234 Dako) were used. For visualizing primary rabbit antibodies, both the 3' phosphothioadenosine 5'-phosphosphate (PAP) method and the biotin-streptavidin method were used. Rat primary antibodies were demonstrated with peroxidase-conjugated rabbit anti-rat IgG (1:50; P0450 Dako) and 0.6% diaminobenzidine (Sigma) in PBS with 0.02% H₂O₂. Control sections were incubated with irrelevant rabbit and rat antibodies.

**Fluorescein isothiocyanate-dextran labeling of macrophage-like cells.** This technique was described previously (28). Normal adult albinos (BALB/c) mice, W/+ , and W/W* mice received 0.2 ml iv or ip of a solution of 1.42 mM fluorescein-labeled fluorescein isothiocyanate (FITC)-dextran in 0.9% NaCl, 1 day before death.

**Vital methylene blue staining.** The entire intestines from adult albinos (BALB/c) mice, W/+ mice, and W/W* mice, including the FITC-dextran-injected animals, were subjected to vital methylene blue staining under controlled conditions (29), including alternation among the three types of mice with respect to consecutive incubations (using the same batch of medium and on the same day). The animals were killed by cervical dislocation, after which the intestines were carefully isolated, immersed for 1 min at room temperature in 7 × 10⁻⁴ M lecithin (Sigma) in PBS (10 mM NaH₂PO₄, 3 mM KH₂PO₄, and 125 mM NaCl, pH 7.3), followed by incubation for 45 min at room temperature in subdued light in Tyrode.
solution (in mM: 137 NaCl, 2.7 KCl, 0.36 NaH₂PO₄, 11.9 NaHCO₃, 1.8 CaCl₂, 0.5 MgCl₂, and 5 glucose, pH 7.3) containing 50 μM methylene blue (all chemicals from Merck), and continuously equilibrated with 5% CO₂-95% O₂. The tissues were fixed with aldehyde containing picrate for precipitation of methylene blue (2% glutaraldehyde, 2% formaldehyde, 0.1 M phosphate buffer, and 0.2% picric acid, pH 7.3). After 5 min, the network of myenteric ICC could be identified under a stereomicroscope. Results were confirmed by inspection of whole mounts at higher magnification (Leitz Orthoplan photomicroscope; equipped with an HBO 200-W lamp, a Leitz Plenompax 2, and the relevant Leitz filter block for simultaneous detection of FITC-dextran fluorescence).

Electron microscopy

After overnight aldehyde fixation (2% glutaraldehyde, 2% formaldehyde, 0.1 M phosphate buffer, and 0.2% picric acid, pH 7.3), tissue pieces (2 x 2 mm; duodenum, jejunum, and ileum) were cut from all methylene blue- and FITC-dextran-stained intestines, as well as from nonincubated intestines, and processed for electron microscopy (EM). Specimens were washed with 0.1 M phosphate buffer and postfixed for 1 h in 2% osmic acid and 0.1 M phosphate buffer. After dehydration in a graded series of ethanol, the tissue was block stained for 1 h in 1% uranyl acetate in absolute ethanol and then through propylene oxide to Epon (Merck). One-micrometer sections were stained with toluidine blue to locate suitable areas. Ultrathin sections were cut and stained with alcoholic uranyl acetate and lead citrate and examined in a Philips 300 electron microscope.

Quantification of cells and cellular relations by EM. We performed a quantitative EM analysis of cells and structures sandwiched between the longitudinal and circular muscle layers. First, cell counts of ICC, fibroblast-like cells (FLC), macrophage-like cells (MLC), pericytes, larger cellular processes (from ICC, FLC, MLC), and other structures (capillaries, ganglia, nerves) were related to numbers of cross-sectioned smooth muscle profiles bordersing the site of Auerbach's plexus. Second, we registered the percentage contribution of the same profiles of cross-sectioned smooth muscle cells bordering on the nerve- and muscle-like structures or bordering directly on smooth muscle cells of the other muscle layer. Counts were made on one complete section from each of the following: 1) 42 tissue blocks from W/W* mice (n = 5); 2) each of 20 blocks from W/+ mice (n = 4); and 3) each of 36 blocks from normal albino (BALB/c) mice (n = 4). For each mouse type, specimens from duodenum, jejunum, and ileum were equally represented. Student's t-test was performed to compare the differences in morphology between W/W* and control mice.

Because the deep muscular plexus (DMP) and the ICC associated with the DMP are very regularly organised, with components mainly following a course parallel to the cells of the circular muscle layer, cross-sectioned profiles of complexes of ICC and nerve fascicles were counted in all those specimens that had the cross-sectioned circular muscle.

Criteria for identification of the cell types normally present in the interval between the muscle layers have been established in earlier studies (35, 40). In brief, ICC associated with Auerbach's plexus (ICC-AP) had oval, rather electron-lucid nuclei, with scanty perinuclear cytoplasm, except for the origins of primary processes. Processes had an abundance of mitochondria, intermediate and thin filaments, microtubules, and endoplasmic reticulum (ER) mainly of the smooth type. The plasma membrane needed a significant number of caveolae profiles of cell bodies and processes to be identified as ICC (35, 40). A basal lamina was incomplete or inconspicuous. The processes generally overlapped to form thin bundles, with close membrane apposition and scattered, small gap junctions. ICC-AP processes typically were interposed between longitudinal muscle and the ganglia and primary fascicles of the myenteric plexus. In addition, ICC-AP partially enveloped MLC. FLC had no basal lamina, no caveolae, and ER primarily of the rough type, typically with somewhat dilated rough ER cisternae. Its nuclear appearance was similar to ICC, with perinuclear cytoplasm often more abundant, and a fair number of mitochondria. FLC processes generally showed up as very thin elongated profiles with few or usually no mitochondria and inconspicuous filamentous contents. MLC could generally be identified by their more electron-dense nuclei, less differentiated cytoplasm with lysosomal granules of variable sizes and coated vesicles, the plasma membrane-like FLC being devoid of caveolae but exhibiting frequent coated invaginations. Profiles of MLC processes were short and blunt, compared with ICC and FLC. After FITC-dextran, MLC contained numerous very large lysosomal vesicles. Pericytes shared a number of ultrastructural features with ICC-AP (filaments, caveolae, basal lamina), however, pericytes had a much higher density of caveolae, had very distinct and complete basal laminae, and were always located at very close proximity to a capillary.

RESULTS

Characteristics of Electrical Activities in +/+ and W/+ + Mice

The small intestine of genetic control mice (+/+ and W/+ +) generated electrical activities that were not distinguishable from albino controls (i.e., CD1) (Table 1, Fig. 2). In all cases, tissues from albino controls (n = 17), W/+ (n = 2), and +/+ (n = 18) generated omnipresent slow-wave-type action potentials. Slow waves occurred at a very constant frequency of ~35 cycles/min in +/+ and in CD1, arising from a resting membrane potential (RMP) of ~62 mV in +/+ and ~58 mV in albino controls (Table 1). Slow-wave frequency was independent of the resting membrane potential (Fig. 2C; r² = 0.06). In cases where spiking activity was present, it occurred superimposed on the plateau phase of the slow waves (Fig. 2).

Blockade of K⁺ channels with TEA (10 mM) or Ba²⁺ (0.5 mM) increased the amplitude of the spiking activity (Fig. 3, Table 2). Other parameters of the electrical activity including the slow-wave frequency, amplitude, duration, and the RMP were not significantly affected by TEA (Table 2), although in two of six experiments TEA decreased the RMP by 6 and 9 mV. The effect of Ba²⁺ (0.5 mM) was similar to that of TEA (10 mM) (Fig. 3B).

The spiking activity in +/+ mice was very sensitive to L-type Ca²⁺ channel blockers such as verapamil and nifedipine, as previously described (16, 25). After application of verapamil, the spiking activity disappeared within 15 min of perfusion. Other characteristics of the slow waves were not affected (Table 1). The L-type Ca²⁺ channel-insensitive component (or the upstroke potential) exhibited sensitivity to extracellular calcium. Perfusion with Ca²⁺-free Krebs (n = 5) in the presence of verapamil markedly reduced or abolished the slow-wave-type action potentials (Table 3). This effect, how-
Table 1. Action potentials in Krebs and verapamil in W/W<sup<v</sub> and control mice

<table>
<thead>
<tr>
<th></th>
<th>W/W&lt;sup&gt;v&lt;/sup&gt;</th>
<th>+/+ (W/W&lt;sup&gt;v&lt;/sup&gt;)</th>
<th>CD1 Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Krebs</td>
<td>Verapamil</td>
<td>Krebs</td>
</tr>
<tr>
<td>n</td>
<td>20</td>
<td>17</td>
<td>14</td>
</tr>
<tr>
<td>Resting membrane potential, mV</td>
<td>-48.1 ± 1.2</td>
<td>-45.2 ± 1.5</td>
<td>14</td>
</tr>
<tr>
<td>Amplitude of slow component (W/W&lt;sup&gt;v&lt;/sup&gt;) or slow wave (+/+; CD1), mV</td>
<td>10.2 ± 0.7</td>
<td>0</td>
<td>18.4 ± 1.7</td>
</tr>
<tr>
<td>Duration, s</td>
<td>0.54 ± 0.05</td>
<td>0</td>
<td>0.78 ± 0.04</td>
</tr>
<tr>
<td>Average rate of rise of the upstroke, mV/s</td>
<td>62.3 ± 7.3</td>
<td>0</td>
<td>97.6 ± 12.7</td>
</tr>
<tr>
<td>Frequency of action potentials, cycles/min</td>
<td>15.1 ± 2.4</td>
<td>0</td>
<td>35.1 ± 1.2</td>
</tr>
<tr>
<td>Amplitude of spike component, mV</td>
<td>10.9 ± 1.2</td>
<td>0</td>
<td>11.1 ± 1.8</td>
</tr>
<tr>
<td>Frequency of spikes, spikes/s</td>
<td>6.3 ± 0.5</td>
<td>0</td>
<td>5.4 ± 0.7</td>
</tr>
<tr>
<td>Average rate of rise of spike, mV/s</td>
<td>242 ± 30.5</td>
<td>0</td>
<td>218 ± 44.2</td>
</tr>
<tr>
<td>Spikes per action potential</td>
<td>2.4 ± 0.2</td>
<td>0</td>
<td>2.3 ± 0.1</td>
</tr>
<tr>
<td>Amplitude of hyperpolarization, mV</td>
<td>5.2 ± 0.5</td>
<td>0</td>
<td>2.3 ± 0.1</td>
</tr>
<tr>
<td>Average rate of rise of depolarization, mV/s</td>
<td>6.3 ± 1.0</td>
<td>0</td>
<td>6.3 ± 1.0</td>
</tr>
<tr>
<td>Quiescence</td>
<td>10/30</td>
<td>0</td>
<td>0/18</td>
</tr>
</tbody>
</table>

Values are means ± SE. *Includes quiescent preparations; excluding quiescent preparations the value is 45.2 ± 1.5 (n = 20). **P < 0.01, significant difference with respect to W/W<sup>v</sup> parameters (unpaired t-test). ***P < 0.05, significant difference with respect to the parameters in Krebs (unpaired t-test). **P < 0.05, significant difference with respect to W/W<sup>v</sup> parameters (unpaired t-test). *Includes only spiking slow waves.

However, was not immediate. In some cases slow-wave activity persisted even after 15 min of perfusion but at a much reduced amplitude, rate of rise, and frequency. Ni<sup>2+</sup> (up to 2 mM) failed to completely block the slow wave but did have pronounced effects. It reduced the slow-wave amplitude, frequency, and rate of rise as well as increased the slow-wave duration (Table 3). These changes occurred independent of membrane depolarization, although over time tissues depolarized in the presence of Ni<sup>2+</sup>. TTX (1 μM; n = 3) had no effect on the upstroke potential in +/+ mice.

Characteristics of Electrical Activities of W/W<sup>v</sup> Mice

Figure 2B illustrates three types of electrical activities that were identified in W/W<sup>v</sup> mice (n = 30). The top tracing shows the most common type of activity (n = 14). The average RMP in this group was -43.6 ± 1.0 mV (range: -38 to -50 mV). Action potentials usually arose at irregular frequency. The action potentials consisted of a relatively slow component (6–10 mV) with superimposed faster spiking activity (Figs. 1 and 2). They were preceded by a slow depolarization and followed by a transient hyperpolarization below the RMP (Figs. 1B, 2B, and 4). A second type of activity (n = 6) showed similar action potentials but occurred in bursts of two to four followed by a period of quiescence of 15.0 ± 3.1 s duration (range: 7.0–29.0 s; n = 6, Fig. 2B). The average RMP in this group was -46.1 ± 1.6 mV (range: -40 to -52 mV). Within the burst interval the action potential frequency reached 40.2 ± 2.4 cycles/min. The bottom tracing of Fig. 2B shows the third type of activity, a lack of action potentials, i.e., electrical quiescence (n = 10). The average RMP in this group was -54.9 ± 1.6 mV (range: -64 to -48 mV). The tissues that exhibited quiescence or very low amplitude oscillations could be induced to show action potentials, similar in appearance to spontaneous action potentials in other W/W<sup>v</sup> preparations, when superfused with BaCl<sub>2</sub> (n = 8; Fig. 4A) or TEA (n = 2). In 6 of 8 experiments, addition of 0.5 mM Ba<sup>2+</sup> depolarized the cell by 2–16 mV giving an average RMP of -49.8 ± 1.5 mV (P < 0.05; control prior to Ba<sup>2+</sup>: 55.5 ± 2.2 mV).
Fig. 3. K⁺ channel blockade in +/- mice. A: effect of K⁺ channel blockade with 10 mM tetraethylammonium chloride (TEA) on slow-wave-type action potentials. B: effect of K⁺ channel blockade with 0.5 mM Ba²⁺ on slow-wave-type action potentials. Drugs were added at 1st arrow and removed at 2nd arrow. Both tracings are parts of a continuous recording from a single cell with lapses of 1–5 min between tracings. Underlined portions were recorded at a ×10 higher chart speed.

\( n = 8 \), significantly lower than that of control quiescent activity. Figure 2C shows that in W/W⁺ mice there is a weak linear relationship between the action potential frequency and the RMP \((r^2 = 0.40)\), with tissues showing quiescence at the most hyperpolarized membrane potentials. There was a large degree of variability in the RMP and action potential frequency recorded in W/W⁺ tissues.

K⁺ channel blockade with 10 mM TEA produced a number of effects on the action potentials in W/W⁺

<p>| Table 2. Sensitivity of action potentials in +/ + and W/W⁺ mice to 10 mM TEA |
|---------------------------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th></th>
<th>W/W⁺ ( n = 9 )</th>
<th>+/+ ( W/W⁺ ) ( n = 6 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting membrane potential, mV</td>
<td>-44.4 ± 1.2</td>
<td>-62 ± 2.3</td>
</tr>
<tr>
<td>Amplitude of slow component ((+/+, CD1)), mV</td>
<td>7.6 ± 1.0</td>
<td>19.1 ± 2.0</td>
</tr>
<tr>
<td>Duration, s</td>
<td>0.44 ± 0.06</td>
<td>0.64 ± 0.07</td>
</tr>
<tr>
<td>Average rate of rise of the upstroke, mV/s</td>
<td>55.9 ± 13.9</td>
<td>97.5 ± 16.6</td>
</tr>
<tr>
<td>Frequency of action potentials, cycles/min</td>
<td>18.0 ± 2.3</td>
<td>38.0 ± 13.3</td>
</tr>
<tr>
<td>Amplitude of spikes component, mV</td>
<td>11.1 ± 2.8</td>
<td>9.7 ± 2.6</td>
</tr>
<tr>
<td>Amplitude of hyperpolarization, mV</td>
<td>4.0 ± 0.9</td>
<td>3.2 ± 0.6</td>
</tr>
<tr>
<td>Average rate of rise of depolarization, mV/s</td>
<td>5.3 ± 1.7</td>
<td>21.0 ± 1.3</td>
</tr>
</tbody>
</table>

Values are means ± SE. TEA, tetraethylammonium chloride. \* \( P < 0.05 \), \# \( P < 0.01 \), significant effects in comparison with values in Krebs (paired t-test). \& \( P < 0.05 \), \& \( P < 0.01 \), significant effects in comparison with values in W/W⁺ mice (unpaired t-test).
Table 3. Effect of Ca²⁺-free Krebs and Ni²⁺ on the slow waves in presence of verapamil in +/- +/+ mice

<table>
<thead>
<tr>
<th></th>
<th>+/+ (n = 5)</th>
<th>+/- (n = 5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Zero Ca²⁺</td>
</tr>
<tr>
<td>Resting membrane potential, mV</td>
<td>57.5 ± 4.8</td>
<td>52.0 ± 4.9</td>
</tr>
<tr>
<td>Slow-wave amplitude, mV</td>
<td>18.4 ± 2.0</td>
<td>22.8 ± 2.9</td>
</tr>
<tr>
<td>Slow-wave frequency, cycles/min</td>
<td>34.2 ± 3.8</td>
<td>14.2 ± 2.5</td>
</tr>
<tr>
<td>Slow-wave duration, s</td>
<td>0.69 ± 0.09</td>
<td>0.59 ± 2.8</td>
</tr>
<tr>
<td>Average rate of rise of the upstroke, mV/s</td>
<td>76.9 ± 16.1</td>
<td>8.9 ± 6.0*</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th></th>
<th>+/+ (n = 5)</th>
<th>+/- (n = 5)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>2 mM Ni²⁺</td>
</tr>
<tr>
<td>Resting membrane potential, mV</td>
<td>56.0 ± 4.0</td>
<td>46.8 ± 2.0*</td>
</tr>
<tr>
<td>Slow-wave amplitude, mV</td>
<td>20.4 ± 1.0</td>
<td>6.0 ± 1.8*</td>
</tr>
<tr>
<td>Slow-wave frequency, cycles/min</td>
<td>36.0 ± 3.0</td>
<td>21.4 ± 4.3*</td>
</tr>
<tr>
<td>Slow-wave duration, s</td>
<td>0.66 ± 0.07</td>
<td>1.04 ± 0.15*</td>
</tr>
<tr>
<td>Average rate of rise of the upstroke, mV/s</td>
<td>105.0 ± 21.8</td>
<td>8.7 ± 1.5*</td>
</tr>
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Values are means ± SE. *P < 0.05, †P < 0.01, significant values in comparison with control values (paired t-test).

The common effect observed in all cases was an increase in the amplitude of the spike component (Table 2). The action potential frequency also increased in most experiments (6 of 8), with no effect on the duration of the slow component in these experiments. In the two experiments in which the action potential frequency did not increase, the duration of the slow component increased as well as the number of spikes superimposed onto the slow component (Fig. 4E). In six experiments in which the slow component had a low average rate of rise (10–40 mV/s), the rate of rise increased 50–275%. When action potentials appeared in bursts, TEA increased the number of action potentials per burst with the bursting activity remaining irregular (Fig. 4C). In experiments with very high action potential frequency in TEA (>50 cycles/min), the action potential consisted of a single spike superimposed on a short-duration slow component (~0.25 s; Fig. 4D). Depolarization was not a prerequisite of any of the observed effects, although two of eight experiments showed a decrease in the membrane potential by 4 and 10 mV.

The action potentials of W/W' mice were almost instantly abolished if one of the following was added to the perfusing solution: 5 × 10⁻⁴ M verapamil (n = 17, Table 1), Ca²⁺-free solution (n = 5), or 2 mM NiCl₂ (n = 6). Verapamil abolished all the electrical activity within 15 min of perfusion (Table 1). In five experiments in which the effect of Ca²⁺-free solution was determined, the average resting membrane potential changed from −44.0 ± 1.9 to −43.6 ± 1.9 mV (no significant difference), whereas the action potentials were completely abolished within 30 s of perfusion. The control data were slow component amplitude, 9.6 ± 1.5 mV; average rate of rise of slow component, 58.0 ± 13.6 mV/s; duration, 0.5 ± 0.1 s; frequency of the action potentials, 15.6 ± 3.8 cycles/min; amplitude of hyperpolarization, 5.0 ± 0.9 mV; and spike amplitude, 10.7 ± 1.5 mV. In six experiments in which the effect of 2 mM NiCl₂ was determined, the action potentials were abolished within 30 s of perfusion, whereas the RMP was not affected (~45.0 ± 3.0 mV). The control data were RMP, −45.5 ± 2.9 mV; slow-component amplitude, 10.7 ± 1.5 mV; average rate of rise of slow component, 56.7 ± 11.2; duration, 0.5 ± 0.1 s; frequency of action potentials, 20.3 ± 4.8 cycles/min; and spike amplitude, 11.7 ± 2.8 mV. TTX (n = 3) had no effect on the spikelike action potentials.

Ultrastructural Characteristics of ICC, FLC, and MLC in Auerbach's Plexus of the W/W' Intestine

Identification of methylene blue-positive cells. In all control mice (albinos, +/-, and W/+) a complete network of ICC-AP was strongly stained with methylene blue as described previously (18). We now subjected these tissues to EM. The firm distinction between interstitial cells of Cajal, FLC, MLC, and pericytes (see methods) allowed us to identify the methylene blue-positive cells as ICC. Although methylene blue was
extracted from the tissue during dehydration, the previously stained cells retained an altered ultrastructure. The chromatin became very dark and finely granular, and ribosomes appeared as finely granular particles, smaller than normal, somewhat similar to nuclear chromatin (Fig. 5). In control and W/W<sup>v</sup> mice, all cells and processes that had this changed ultrastructure also had the characteristic of interstitial cells of Cajal of Auerbach's plexus, to the exclusion of FLC, MLC, and pericytes. In W/W<sup>v</sup> mice, only very few and scattered ICC were stained in the Auerbach's plexus area, with no evidence of a communicating network, as reported previously (18). In sections from methylene blue-stained W/W<sup>v</sup> mice, a length of the Auerbach's plexus region corresponding to a total of 2,196 profiles of cross-sectioned border smooth muscle cells, a total of 9 ICC cell bodies and 12 larger processes of ICC were identified. The ultrastructure of smooth muscle cells was normal except for the appearance of dumbbell-shaped mitochondria (Fig. 6), despite the fact that normal contacts with ICC were absent. Corresponding to the same number of smooth muscle cell profiles, methylene blue-stained control mice had 95 ICC cell bodies and 975 profiles of larger processes of ICC.

Were ICC the only cells affected by the W mutation? The main structural questions to be answered were whether the ICC network in W/W<sup>v</sup> was simply absent, whether it was replaced by other cells such as FLC, or whether there was still a network of underdeveloped ICC not recognized by EM as ICC. To answer these questions we first did a quantitative analysis of cells at
the borders between the muscle layers (Fig. 7). In control mice, for every 100 smooth muscle cells counted ~4.3 ICC were in close apposition to a smooth muscle cell in BALB/c and W/+ mice and 0.2 ICC in W/W0 mice (P < 0.001). Compared with W/+ mice, the number of FLC in W/W0 mice increased 75% from 2.2 to 3.8 per 100 smooth muscle cells (P < 0.001) and MLC increased 183% from 0.6 to 1.7 per 100 muscle cells (P < 0.001). The number of capillaries, ganglia, pericytes, primary, secondary, and tertiary nerve fascicles were not different between W/W0 and controls (Fig. 7). MLC, ganglia, and primary fascicles were enveloped by ICC in control but never in W/W0 mice.

To further investigate how the environment of smooth muscle cell had changed, the percentage of smooth muscle cells bordering on other structures was measured (Fig. 8). As expected, the percentage of smooth muscle cells bordering on ICC went down from 55 to 1% in W/W0 mice (P < 0.001). Interestingly, the percentage of smooth muscle cells bordering on muscle cells from the opposite muscle layer (i.e., circular bordering longitudinal muscle) increased 180% from 15 to 42% (P < 0.001). The number of smooth muscle cells bordering on MLC went up 340% compared with W/, from 1.5 to 6.6% (P < 0.001), whereas that of smooth muscle cells bordering on FLC went up 340%, from 6 to 22% (P < 0.001).

Inspection of toluidine blue-stained 1-μm sections did not reveal any differences in overall organization of the muscle layers or in distribution of profiles of myenteric ganglia and fascicles at the light microscopy level. The structure of the myenteric plexus was further examined in whole mounts after immunostaining with antibody against GFAP, a glial marker protein (3, 19, 32). Figure 9 shows the normal appearance of Auerbach's plexus in W/W0 mice. The absence of ICC had little or no influence on the development of the glial and neural structures.

In W/W0 mice, reticulocytes with ribosomes were seen in the capillary lumen (Fig. 10). It would be most unusual to find this in a normal animal, and it is likely associated with the anemia of the W mutant mouse.

FLC. FLC are increased in number in W mutant mice in the area where normally the ICC network is located. Figure 11 shows an FLC bordering on both the longitudinal and circular muscle cells, easily distinguished by dilated rough ER reticulum, very large Golgi areas, absence of caveolae and basal laminae. The FLC did not accumulate methylen blue. Although the number of FLC increased markedly, the percentage of smooth muscle profiles bordering on FLC in W/W0 mice was much smaller than the percentage of smooth muscle cells bordering on ICC in control tissues. The typical spatial relations that ICC in normal mice have to ganglia and nerve fascicles (forming a partial sheath),
to MLC (partial envelopment), and to smooth muscle cells (forming conspicuous peg and socket junctions with cells of the longitudinal muscle layer) were not characteristics of the FLC in W/W<sup>υ</sup> mice.

**MLC.** MLC associated with muscle cells bordering the myenteric plexus were increased in number compared with controls. The location of the MLC shown in Fig. 10, bordering a primary nerve fascicle, a longitudinal, and a circular muscle cell, and a capillary, invariably would have housed an ICC in normal tissue. In two mice that on the previous day had been injected with fluorescence-labeled FITC-dextran, the assembly of MLC in the muscle layers was normal. In all mice, characteristics and organization of the MLC at the Auerbach's plexus level and elsewhere were normal, except that MLC were not enveloped by ICC processes. The MLC did not form a network.

**Deep Muscular Plexus**

It was of great interest to see whether the ICC in the deep muscular plexus were affected in the W mutant mice. All ultrastructural features of cross-sectional complexes of nerve fascicles and ICC were normal. The density of these complexes was also normal: 21.0/100 cross-sectional smooth muscle cells in BALB/c, 20.3/100 in W/+; and 21.3 in W/W<sup>υ</sup> mice.

**Kit immunoreactivity.** In albino mice and W/+ genetic controls, Kit immunoreactivity was present in cells between the circular and longitudinal muscle at the level of Auerbach's plexus, its distribution pattern typical for the network of ICC as reported previously (18). In addition, immunoreactivity was present in cells associated with the DMP (Fig. 12). The distribution was consistent with that of the network of ICC in this location (35, 40). The distribution cannot be compared with that of methylene blue staining because ICC in the DMP do not accumulate methylene blue. Of the two antibodies, the polyclonal antibody against an intracellular epitope produced the stronger immunoreactivity. In W/W<sup>υ</sup> mice and in controls with irrelevant antibodies, anti-Kit immunoreactivity was not detected.

**DISCUSSION**

Action potentials in W/W<sup>υ</sup> mice exhibited the following characteristics. Superimposed on a relatively slow component (~10 mV), faster spiking activity occurred (Figs. 1, 2, and 4). The action potentials were preceded by a slowly developing depolarization and followed by a transient hyperpolarization to ~5 mV below the RMP. The amplitude including spikes was ~20 mV. The action potential frequency, amplitude, and duration were markedly variable, and in quiescent tissues the activity could be evoked by inhibition of K<sup>+</sup> conductance. The activity was completely and quickly abolished by the L-type Ca<sup>2+</sup> channel blockers verapamil and nifedipine. The variability in action potential frequency was weakly linearly related (r<sup>2</sup> = 0.40) to variability in the RMP (Fig. 2C). The differences in activity between +/- and W/W<sup>υ</sup> mice were not due to their difference in the RMP. Many tissues in W/W<sup>υ</sup> mice exhibited RMPs similar to that of +/- mice. The most hyperpolarized tissues were usually electrically quiescent; however, they exhibited typical W/W<sup>υ</sup> action potentials on K<sup>+</sup> channel blockade with an associated depolarization of the cells.

The action potentials described above are very similar to action potentials generated by the circular muscle layer of the canine colon from which the ICC network has been removed (22). Similarly, the isolated outer circular muscle strips from the dog small intestine (12) separated from ICC were electrically quiescent, and acetylcholine generated slow potentials with spikes preceded by a diastolic-type depolarization (13). In the cat small intestine (6), after abolishment of slow waves by ouabain, acetylcholine induced electrical activity that very closely resembled the activity seen in W/W<sup>υ</sup> small intestine; slow potentials with spikes, preceded by a prepotential and followed by transient hyperpolarizations; the action potentials were abolished by L-type Ca<sup>2+</sup> channel blockers (6). Importantly, action potentials with many if not all of the features recorded from the W/W<sup>υ</sup> mouse, including insensitivity to TTX, were recorded from chemically isolated smooth muscle cells from the dog colon (33), rabbit small intestine (1), and mouse small intestine (J. C. F. Lee and J. D. Huizinga, unpublished observations). With all of the above evidence taken into account, it is our hypothesis that the action potentials generated in the W/W<sup>υ</sup> mouse small intestine are generated by their smooth muscle cells and, in fact, reflect intrinsic electrical activity of smooth muscle cells. In W/W<sup>υ</sup> mice, TTX and Ba<sup>2+</sup> increased the amplitude and frequency of action potentials and induced burst-type activities. In control mice, only the intensity of spiking activity, superimposed on the slow waves, was increased by K<sup>+</sup> channel blockade. This illustrates how the slow waves restrict the range of
Fig. 11. FLC in W/W<sup>+</sup> mice did not replace the ICC network. Electron micrographs of distal ileum of a W/W<sup>+</sup> mouse. Ultrastructurally normal FLC were sandwiched between the two muscle layers. Longitudinal (LM) and circular muscle (CM) cells appeared completely normal. Cytoarchitecture of interstitial tissue between the muscle layers was generally simplified due to deficiency of extensively branching ICC. None of the typical structural contacts that ICC have with bordering cells were identified with FLC. B: magnification of area well-developed rough endoplasmic reticulum (rer) in FLC. g. Golgi apparatus. Magnification: ×10,000 (A) and ×37,000 (B).

activities the smooth muscle cells carry out in tissues.

Whether activity is recorded in tissues from W/W<sup>+</sup> mice depends sizably on

Fig. 12. Mouse small intestine has anti-Kit antibodies, accompanying components of Auerbach’s plexus as well as in cells at the deep muscularis. Experiment was done on frozen unfixed mouse. Magnification ×355. See METHODS for details.
lene blue, is absent in W/W<sup>+</sup> mice (18), schematically represented in Fig. 13. However, scattered methylene blue-positive cells remained. The present study shows by EM investigation that these cells are ICC and not precursor cells or another cell type "replacing" ICC. Occasionally, spaces normally occupied by ICC were seen to house FLC or MLC. In such cases, the FLC or MLC did not make the strong contacts with neighboring smooth muscle and nerve cells that ICC normally do. Furthermore, spaces normally occupied by ICC were most often absent, and the longitudinal and circular muscle layers directly apposed each other (Fig. 7). The remaining ICC did not form a network with the FLC or MLC, nor did these latter cell types form a network by themselves. It is of course significant that the number of FLC increased markedly in W/W<sup>+</sup> mice; whether this relates to FLC being precursor or underdeveloped ICC cannot be determined at present, but it is unlikely because they have the typical features of normal FLC. The reason why they are increased in W/W<sup>+</sup> remains unclear.

No firm answer can be given to the question whether the abnormal kit gene resulted in alterations of the smooth muscle cells. Structurally, there is little evidence for a change, except for the appearance of dumbbell-shaped mitochondria. This could indicate an increased number of dividing mitochonridia due to anemia. Electrophysiologically, the smooth muscle cells generate action potentials similar to smooth muscle tissues not governed by slow-wave pacemaker activity, i.e., colonic longitudinal muscle (5, 21) and tissue from which ICC were mechanically removed (6, 12, 13, 22). However, smooth muscle tissue in W/W<sup>+</sup> mice is depolarized compared with +/- mice. This could be a direct consequence of the absence of ICC, i.e., on physical removal of the submuscular ICC from colonic circular smooth muscle, the muscle cells depolarize (21). Similarly, in the cat small intestine, longitudinal muscle cells depolarize on removal of ICC (39). However, the RMP of circular muscle cells of some other species does not show significant change when ICC are cut away (12, 39). At this time, an actual change in smooth muscle primarily due to the alteration in the kit gene cannot be excluded.

In control tissues, the Kit protein was identified in the network of ICC-AP (16, 43); the present study confirms its presence around the DMP. In W/W<sup>+</sup> mice, the ICC network at the DMP site is present (ICC-DMP), identical to that in control tissue, but without the normal Kit protein. Hence, the ICC-DMP do not need the Kit protein for normal development. From the present study it can also be concluded that the ICC-DMP in the mouse small intestine are not involved in the generation of pacemaker activity. These data add to a growing awareness that the ICC networks in the gastrointestinal tract are diverse both in function and in morphology. Several differences between ICC-AP and ICC-DMP, in addition to a dependence on the Kit protein, are known (35, 40). ICC-DMP have a very strong connection through gap junctions with smooth muscle cells, whereas the ICC-AP are connected through close apposition contacts. ICC-DMP have a complete basal lamina, whereas that of ICC-AP is patchy. In contrast to ICC-AP, ICC-DMP do not accumulate methylene blue; because methylene blue is accumulating in the ER, there are differences in the ER between ICC-AP and ICC-DMP. Interestingly, Ca<sup>2+</sup> from the ER is implicated as a crucial factor in the generation of slow waves (23). Hara et al. (12), studying the small intestine of various mammals, describe spontaneous activity in the inner circular muscle layer that is associated with the DMP but mechanically separated from the bulk of the circular muscle and from longitudinal muscle and the AP. This could be interpreted as suggesting the generation of pacemaker activity (typical slow wave) by the ICC-DMP. However, Hara et al. (12) carefully describe the activity as slow potentials with spikes, not slow waves. Furthermore, the amplitude of the slow potential is variable from 0 to 10 mV, and the frequency is variable from 0 to 18 cycles/min and preceded by a "slow diastolic-type" depolarization. This is in contrast to the activity recorded from the same site in intact tissue: amplitude 17 ± 2 mV, frequency 13 ± 1 cycles/min. Hence, the activity recorded in isolated inner circular muscle very much resembles "smooth muscle activity," i.e., similar to W/W<sup>+</sup> activity and smooth muscle tissue from which ICC are removed. It would be very interesting to learn the sensitivity of this activity to L-type Ca<sup>2+</sup> channel blockers.

It is our hypothesis that the absence of ICC and decreased transit in W/W<sup>+</sup> mice are causally related. As early as 1960, Kojima et al. (20) reported that intestinal transit was slow in W/W<sup>+</sup> mice. Ishikawa et al. (17) observed that worm expulsion after a primary Nippostrongylus brasiliensis infection was completed by day 10 compared with day 7 in +/- mice. Also, when 150 N. brasiliensis adult worms were implanted intraduodenally 100 ± 8 (n = 4) remained in the small intestine after 3 days in W/W<sup>+</sup> mice, whereas only 30 ± 2 remained in the +/- mice. Bone marrow transplant did restore the mast cell population but did not restore transit to normal. In Ws/We rats, also deficient in ICC

![Fig. 13. Schematic representation of structural changes occurring in a W/W<sup>+</sup> mouse. G and NF, ganglion and nerve fascicle. For other abbreviations, see Figs. 7, 8, and 11.](image-url)
and pacemaker activity in the small intestine (18), dramatic bile reflux into the stomach was noted. The question remains how intestinal contractile activity in W/W* mice is organized to move contents anally. We hypothesize, based on preliminary evidence (15), that in W mutant mice, the contractions will be random and that normal peristalsis is lacking. In +/- mice the contractions will be governed by the pacemaker (slow-wave) frequency gradient inducing anally directed propulsive contractions resulting in normal peristalsis.

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REFERENCES


Chapter Five


Methods and contributions

John Malysz performed all electrophysiological experiments, carried out all analysis of electrophysiological data, and was involved in writing the manuscript and in study design. Dr. Lars Thuneberg carried out all light and electron microscopic studies, and Dr. Hanne Mikkelsen performed the anti kit receptor immunohistochemistry. Dr. Jan D. Huizinga initiated the studies, was involved in study design and writing, and coordinated all projects.
Action potential generation, Kit receptor immunohistochemistry and morphology of steel-Dickie (SI/SI^d) mutant mouse small intestine

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Abstract In contrast to wild-type mice, homozygotes with mutations of the W locus do not express the functional Kit receptor and are severely deficient in the Auerbach's plexus (AP)-associated subtype of interstitial cells of Cajal (ICC-AP). With a morphologically intact neural and muscular structure, the absence in these mutants of both small-intestinal slow waves and ICC-AP constitutes strong evidence for a key role of ICC-AP as pacemaker cells. In steel-Dickie mutant mice (SI/SI^d), the gene coding for the Kit ligand (stem cell factor) is defective. We examined SI/SI^d mutants and controls with intracellular microelectrode techniques, combined with light and electron microscopy. The absence of the normal Kit ligand (SI/SI^d mice) had very similar effects as the absence of the Kit receptor in viable mice, mutated at the White spotting, W, locus (W/W^m mice), in that neither slow waves, nor Kit receptor immunoreactivity in the region of Auerbach's plexus nor ICC-AP were present in the small intestine. In the SI/SI^d mouse, the smooth muscle cells generated action potentials at variable frequencies from a depolarized cell membrane of ~40 to ~55 mV. Increasing excitability by K channel blockers created many different patterns of action potential generation and the frequency increased from ~16 cpm to 66 cpm. This was in sharp contrast to control mice where action potentials were always restricted to the plateau phase of the slow waves and the slow wave frequency remained constant at ~39 cpm. Our data provide further strong support for the identification of ICC-AP as small-intestinal pacemaker cells. In addition, they provide a basis for the understanding of intestinal motor function without pacemaker activity.

Keywords interstitial cells of Cajal, mouse, pacemaker, small intestine, steel mutation.

INTRODUCTION

The steel-Dickie (SI/SI^d) mutant mouse lacks the membrane-bound form of stem cell factor (SCF) which is the natural ligand for the Kit receptor. The Kit receptor is known to map to the mouse White Spotting (W) locus. Known defects of SI/SI^d mice are similar to those of viable white-spotted (W'W') mutant mice (abnormal pigmentation, anaemia, abnormal development of gametes) which is seen as evidence for an essential interaction between stem factor and the Kit receptor in normal mice. Recent work on W'W' mice has suggested that interstitial cells of Cajal (ICC) in the small intestine were dependent on the Kit receptor for normal development. Maeda et al. reported that newborn mice, receiving injections of an anti-Kit receptor antibody, lost certain Kit-receptor-positive cells, which were normally present in the intestinal external muscle layers. Subsequent studies identified these cells as ICC. Of the two ICC networks present in the small intestine, the one associated with Auerbach's plexus (ICC-AP) was intact, while the other network associated with the deep muscular plexus (ICC-DMP) was intact. Interestingly, ICC in both locations bind anti-Kit antibody, indicating a difference in dependence on the ligand/receptor system. The parallel absence of ICC-AP and electrical slow waves is currently the strongest evidence in favour of the origin of this activity in ICC-AP (hence designated pacemaker cells).
Considering the otherwise similar defects in SI/SI<sup>d</sup> and W/W<sup>""</sup> mutants, we hypothesized that also the ICC and electrical activity might be affected in a parallel way in the two mutants. Our aim was (1) to study by combined light and electron microscopy the quantitative aspects of the cellular organization and (2) to perform a detailed analysis of the electric activity of the steel-Dickie mutants.

This work was published previously in abstract form. While our work was in progress, the results of a parallel study were published; the two studies support and supplement each other.

MATERIALS AND METHODS

Immunohistochemistry

Adult albino mice, SI/SI<sup>d</sup> and SI/® mice were killed by cervical dislocation. Specimens from the small intestine were prepared in a Tyrode’s solution, quick-frozen in isopentane cooled in liquid nitrogen and stored at -80 °C until freeze sectioning.

The immunohistochemical techniques were described previously. Polyclonal rabbit antibodies, and monoclonal rat antibodies (ACK-2; IgG2b, Gibco) against, respectively, the intracellular and extracellular domains of mouse Kit protein tyrosine kinase receptor, were used. The PAP method and the biotin streptavidin Texas Red technique were used to detect primary rabbit antibodies. The peroxidase method and FITC technique were used to detect ACK-2 antibodies. Since the rat antibodies generally gave an unspecific staining of nerves, the sections were, after preincubation with rabbit serum (1:5 in PBS and 1% BSA), incubated with rat primary antibodies (absorbed with mouse serum) diluted with rabbit serum in PBS/BSA overnight. In addition, the second layer antibodies: peroxidase-conjugated rabbit antirat IgG (1:5; PO450 Dako) and FITC-conjugated rabbit antirat IgG (1:40; PO234 Dako) were absorbed with mouse serum. Controls were incubated with irrelevant rabbit antibodies or rat IgG<sub>2b</sub>. We have had to avoid using wholemounts, since in our hands ICC-AP of wholemounts (but not frozen sections) showed identical, however selective, immunoreactivity using specific [Kit receptor] antibody and unspecific, irrelevant antibodies.

Methylene blue staining and FITC-dextran

The entire intestines from albino mice (n = 2), SI/® mice (n = 2) and SI/SI<sup>d</sup> mice (n = 2) were subjected to vital methylene blue staining as previously described. Briefly, the tissue was exposed shortly to 7 x 10<sup>-4</sup> M lysolecithin in PBS followed by incubation in aerated Tyrode containing 50 μM methylene blue for 45 min, until immersion in picrate-containing fixative. All animals subjected to methylene blue staining had on the previous day received an injection, either in a tail vein or intraperitoneally, of fluorescein [FITC]-labelled dextran (0.2 mL of a 1.42 mM solution in 0.9% NaCl, as described previously). The double stained small intestines (methylene blue and FITC-dextran) were evenly stained over their full lengths (FITC-dextran) or in all exposed areas (methylene blue), providing extensive material, including duodenal, jejunal, and ileal regions for inspection.

For fluorescence and bright-field microscopy a Leitz Orthoplan microscope was used.

Electron microscopy

Tissue from all methylene-blue-stained, FITC-dextran-injected mouse intestines, as well as from untreated mice (two Balb/C, two SI/®, three SI/SI<sup>d</sup>), was immersion-fixed (2% glutaraldehyde, 2% formaldehyde, 0.1 M phosphate buffer, 0.2% picric acid, pH 7.3), washed with 0.1 M phosphate buffer, and post-fixed in osmic acid (2% in 0.1 M phosphate buffer, 1 h). The tissue was dehydrated in ethanol, block stained for 1 h in uranyl acetate (1% in absolute ethanol) and taken through propylene oxide to Epon (Merck). Thin sections (1 μm) were stained with toluidine blue, ultrathin sections (50-70 nm) with alcoholic uranyl acetate, followed by lead citrate. The sections were examined and photographed in a Philips 300 electron microscope.

Recording of electrical activities

Adult mice of either sex, SI/SI<sup>d</sup> and ®/+ (to SI/SI<sup>d</sup>) from Jackson Laboratories (Bar Harbor, ME, USA) were killed by cervical dislocation. The small intestine was then exposed by a midline abdominal incision and a segment (5-10 cm) of the ileum was removed with the distal end 1-2 cm away from the cæcum. Segments were placed in a dissecting dish filled with oxygenated (95% O<sub>2</sub> and 5% CO<sub>2</sub>) Krebs solution, opened flat and rinsed for faecal contents. The mucosa was then carefully removed by sharp dissection. Muscle strips, 15 mm long and 5-8 mm wide, were prepared by cutting them parallel to the longitudinal muscle bundles. A muscle strip was mounted on Sylgard and was perfused with Krebs solution at a constant rate (5.0 mL min<sup>-1</sup>) at 36.0–37.0 °C. Intracellular recordings were obtained with microelectrodes of 40-100 MΩ tip resistance, and possessing a tip diameter of ≈ 150 nm. Microelectrodes were prepared from 1.2-mm outside diameter glass.
capillaries (WPI) and filled with 3 m KCl. A microelectrode was inserted into a microelectrode holder (WPI M700P) which connected to an electrometer (WPI M-707A) which is a high-impedance probe. The output of the electrometer was displayed on a Gould oscilloscope and recorded on a Gould inkwriting recorder.

Data analysis of electrical activities
Electrical activity was analysed as previously described in Malysz et al. Briefly, in +/- mice, the following parameters of the electrical activity were measured: the resting membrane potential, slow-wave amplitude, the amplitude of the spike component, the average rate of rise of the upstroke of the slow wave (from 0 to 20 mV), slow-wave duration measured at half maximal amplitude, slow-wave frequency, number of spikes superimposed on a slow wave and spiking frequency. The action potentials of SI/SI* mice were characterized according to the following parameters: resting membrane potential, amplitude of the slow component, amplitude of the spike component, average rate of rise of the slow component (0-5 mV), action potential frequency, slow-component duration measured at half maximal amplitude and amplitude of the hyperpolarization (following the repolarization of the slow component). Data were obtained usually from the same cells, before and in the presence of a drug or after ion or solution substitution. A difference was considered significant if P < 0.05. Data are expressed as mean ± SEM. Paired or unpaired Student’s t-tests were employed for comparisons of the electrical activities. Linear regression analysis was performed on data in order to determine correlation coefficients (r²) between resting membrane potential and action potential frequency for SI/SI* and +/- mice.

Solutions and drugs
The physiological experiments were performed in prewarmed (36-37 ºC) Krebs solution equilibrated with 95%O₂-5%C0₂. The composition of the Krebs solution was (mm): NaCl, 120.3; KCl, 5.9; CaCl₂, 2.5; MgCl₂, 1.2; NaHCO₃, 20.0; NaH₂PO₄, 1.2 and glucose, 11.5. The pH was 7.30-7.35. In experiments where the solution was modified to calcium-free Krebs, all of the ingredients except CaCl₂ were used. In experiments that required verapamil (Sigma) a final concentration in the range of 1-10 x 10⁻⁶ M was obtained. BaCl₂, NiCl₂ and tetraethylammonium chloride (TEA) were obtained from Sigma.

The animals were cared for in accordance with the principles and guidelines of the Danish and Canadian Councils on Animal Care.

RESULTS

Immunocytochemistry
In control mice (albino and SI/+ mice) we found immunoreactivity with both the rabbit (intracellular epitope) and the ACK-2 antibodies (extracellular epitope) in cells which partially enveloped the ganglia and fascicles of Auerbach’s plexus (AP), and in cells associated with the deep muscular plexus (Fig. 1a), with the rabbit antibody a stronger staining was seen with less unspecific background. In SI/SI* mice immunoreactivity was absent at the level of AP with both antibody preparations (Fig. 1b). With one antibody (ACK-2) immunoreactivity was present in cells at the level of the deep muscular plexus (DMP), but consistently absent with the other one (rabbit antibody, Fig. 1b).

Methylene blue and FITC-dextran
Methylene blue staining revealed the characteristic ICC-AP network throughout the small intestine in SI/+ and in albino mice (Fig. 1c), while virtually no ICC-AP were seen in SI/SI* mice (Fig. 1d). Fluorescence microscopy of the FITC-dextran-injected mice showed normal organization and numbers of macrophages in all animals (Fig. 1c,d).

Electron microscopy
In control mice typical ICC-AP were present (Fig. 2). SI/SI* mice lacked ICC-AP (Fig. 3), but ICC-DMP were present and appeared normal (Figs 3a and 4). ICC-AP, when present, were distinguished by their ultrastructure, but also, after methylene blue staining, by the inclusion of a characteristic, finely granular cytoplasmic precipitate. ICC profiles were counted in samples from duodenum, jejunum and ileum from all methylene-blue-stained mice and from two untreated mice of each type. As a reference, cross-sectional profiles of muscle cells bordering on Auerbach’s plexus were counted. Control mice had on average 4.5 ICC-AP cell bodies and 55 profiles of identifiable ICC processes per 100 cross-sectioned neighbouring smooth muscle cells. In control mice this resulted in an average of 56% of such muscle cell profiles bordering on ICC-AP and 15% bordering on smooth muscle cells of the adjacent layer. The corresponding figures for SI/SI* mice were 0% (no ICC or ICC processes could be identified) and 52%, respectively. ICC-DMP in SI/SI* mice were similar to controls, with respect to ultrastructure (nuclear profiles, high numbers of mitochondria, thin and intermediate filaments, surface caveolae), organization (branching patterns, gap junctional coupling between

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Figure 1 (a,b) Rabbit antibody to Kit receptor. (a) Control mouse; Kit receptor immunoreactivity is present in cells enveloping Auerbach’s plexus (AP) between the longitudinal (LM) and circular (CM) muscle layers (arrowhead), and in cells associated with the deep muscular plexus (DMP) between the outer and inner circular muscle layers (arrow). (b) In the SI/SI\(^2\) mutant mice the Kit receptor immunoreactivity with this antibody is absent in both locations. \(\times 415\), bar = 25 \(\mu\)m. (c,d) Wholemounts of isolated muscularis externa from animals injected with FITC-dextran and subjected to vital methylene blue staining. (c) Control mouse (SI/+) with normal appearance of ICC-AP network (stained) and normal organization of macrophages (fluorescence). (d) SI/SI\(^2\) mouse with normal contingent of macrophages, but no ICC-AP network. AP, ganglion of Auerbach’s plexus. The methylene blue-stained structures in (d) are short rows of red blood cells inside capillaries; similar structures are present, but less conspicuous in (c). \(\times 200\), bar = 50 \(\mu\)m.

ICC, as well as between ICC and outer circular muscle, close association with and innervation by varicose axons of DMP, and numbers (in cross-sectioned circular muscle about 20 cross-sectioned ICC/nerve fascicle associations per 100 muscle cell profiles bordering the longitudinal muscle layer). The number and organization of macrophages, fibroblasts and pericytes were normal. SI/SI\(^2\) mice, similar to control mice, had \(\approx 1\) macrophage per 100 cross-sectional profiles of muscle cells (bordering the other muscle layer); macrophages were organized in close association with the outer aspects of Auerbach’s plexus elements, often clearly outlining the primary plexus (Fig. 3b). The only observed deviation from normal ultrastructure, was a change in mitochondrial morphology in smooth muscle cells of both layers: in cross-sectioned profiles dumbbell-shaped mitochondria were frequent (Fig. 4b). These shapes were not observed in control tissue.

Figure 2 Control mice. (a) A nerve (N) is enveloped by typical ICC (arrowheads) between the longitudinal (LM) and circular (CM) muscle layers. The ICC has dark cytoplasm with long cell processes, a high content of mitochondria and many caveolae. \(\times 3900\), bar = 2 \(\mu\)m. (b) An ICC between the longitudinal and circular muscle layers. \(\times 16 200\), bar = 0.5 \(\mu\)m.

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Figure 4 SI/SI² mice. (a) A normal ICC (arrow) at DMP between the outer (top) and inner (bottom) circular muscle layers. × 15 200, bar = 0.5 μm. (b) In the muscle cells a subtle change of mitochondrial morphology was noted (similar to W/W° mice), in that mitochondria with dumbbell-shaped cross-sectional profiles were frequent. × 39 500, bar = 20 μm.

Figure 3 SI/SI² mice. (a) In SI/SI² mice ICC were not identified at the AP site, but the muscle layers, nerve plexus, fibroblasts and macrophages appeared normal. ICC at the deep muscular plexus were normal (arrow). C, capillary. × 3900, bar = 2 μm. (b) A ganglion (nerve cell, N) in continuity with a primary fascicle was present between the muscle layers. M, macrophage; C, capillary. Note the overall absence of a cellular sheath around the ganglion and primary fascicle: normally, ICC ensheathe these structures. × 30 400, bar = 0.5 μm.

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<th></th>
<th>+/+</th>
<th>SI/Sp&lt;sup&gt;+&lt;/sup&gt;</th>
<th>SI/Sp&lt;sup&gt;+&lt;/sup&gt; in 0.5 mM Ba&lt;sup&gt;2+&lt;/sup&gt;</th>
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<tr>
<td></td>
<td>[n=11]</td>
<td>[n=13]</td>
<td>[n=8]</td>
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<tr>
<td>Resting membrane potential (mV)</td>
<td>−58.5 ± 1.9</td>
<td>−49.2 ± 1.8&lt;sup&gt;**&lt;/sup&gt;</td>
<td>−42.9 ± 1.1&lt;sup&gt;**&lt;/sup&gt;</td>
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<td>Amplitude of slow wave (+/+ or slow component [SI/Sp&lt;sup&gt;+&lt;/sup&gt;] (mV)</td>
<td>18.2 ± 146</td>
<td>8.3 ± 1.2&lt;sup&gt;**&lt;/sup&gt;</td>
<td>9.7 ± 0.9&lt;sup&gt;**&lt;/sup&gt;</td>
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<tr>
<td>Spike amplitude (mV)</td>
<td>10.6 ± 1.8&lt;sup&gt;**&lt;/sup&gt;</td>
<td>13.6 ± 1.2</td>
<td>13.8 ± 1.5</td>
</tr>
<tr>
<td>Duration (msec)</td>
<td>0.73 ± 0.04</td>
<td>0.55 ± 0.06&lt;sup&gt;**&lt;/sup&gt;</td>
<td>0.68 ± 0.11</td>
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<td>Average rate of rise of upstroke (+/+ or slow component [SI/Sp&lt;sup&gt;+&lt;/sup&gt;] (mV sec&lt;sup&gt;−1&lt;/sup&gt;)</td>
<td>103.6 ± 10.5</td>
<td>32.8 ± 3.2&lt;sup&gt;**&lt;/sup&gt;</td>
<td>57.5 ± 4.9&lt;sup&gt;**&lt;/sup&gt;</td>
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<td>Action potential frequency (cpm)</td>
<td>38.2 ± 1.6</td>
<td>16.2 ± 1.7&lt;sup&gt;**&lt;/sup&gt;</td>
<td>17.8 ± 2.9&lt;sup&gt;**&lt;/sup&gt;</td>
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<td>Spike frequency (cps)</td>
<td>5.3 ± 0.7&lt;sup&gt;**&lt;/sup&gt;</td>
<td>7.3 ± 0.7</td>
<td>6.7 ± 0.7</td>
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<td>Spikes per slow wave (+/+ or action potential [SI/Sp&lt;sup&gt;+&lt;/sup&gt;]</td>
<td>2.9 ± 0.7&lt;sup&gt;**&lt;/sup&gt;</td>
<td>3.0 ± 0.4</td>
<td>3.2 ± 0.6</td>
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<td>Amplitude of hyperpolarization (mV)</td>
<td>−</td>
<td>4.4 ± 0.8</td>
<td>5.6 ± 0.7</td>
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<tr>
<td>Average rate of rise of depolarization (mV sec&lt;sup&gt;−1&lt;/sup&gt;)</td>
<td>−</td>
<td>5.7 ± 0.9</td>
<td>6.2 ± 1.5</td>
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<sup>**</sup>P < 0.05, <sup>***</sup>P < 0.01 Significant differences between +/+ and SI/Sp<sup>+</sup> mice (unpaired t-test). <sup>†</sup>P < 0.05, <sup>‡</sup>P < 0.01 Significant differences between SI/Sp<sup>+</sup> and SI/Sp<sup>+</sup> in Ba<sup>2+</sup> cells (unpaired t-test). * Includes only spiking slow wave (n=7). † Includes quiescent preparation (n=20); excluding quiescent preparations the value is −46.0 ± 1.4 (n=13).

### Characteristics of electrical activities in +/+ mice

Genetic controls for SI/Sp<sup>+</sup> mice (+/+ ) generated omnipresent slow waves occurring at a very constant frequency within each tissue (Table 1, Fig. 5). In cases where action potentials were present, they occurred superimposed on the plateau phase of the slow waves.

Slow-wave frequency was independent from the resting membrane potential (Fig. 6, r<sup>2</sup> = 0.03).

Action potentials in control mice were very sensitive to blockade with L-type calcium channel blockers such as verapamil (Fig. 7). Following application of verapamil, action potentials disappeared within 10 min of perfusion. Slow-wave characteristics were not affected.
Figure 6  The relationship between action potential frequency and resting membrane potential in +/- and SI/SI'' small intestine. The n numbers for +/-, SI/SI'' and SI/SI'' in 0.5 mM Ba^2+ are, respectively, 11, 20 and seven, and the respective linear correlation coefficients (r^2): 0.03, 0.45 and 0.06. Overlapping experiments are expressed by a single point.

```
  +/+     SI/SI''     SI/SI'' in Ba^2+
```

20 mV

3 μM Verapamil

3 min

5 min

10 min

20 min

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The insensitivity of the slow wave to L-type calcium channel blockers was demonstrated previously in the mouse and rabbit small intestine, and the dog colon. The slow-wave parameters in verapamil (3-5 μM) in control (+/+ or -/-) mice (n = 6) were: resting membrane potential, -58.5 ± 1.3 mV; amplitude, 18.6 ± 1.7 mV; duration, 0.66 ± 0.06 sec; average rate of rise of upstroke, 124 ± 16.9 mV s^-1; and frequency, 39.0 ± 2.8 cpm (cf. Table 1). The slow wave exhibited sensitivity to extracellular calcium. Perfusion of tissues with solutions containing either 2 mM Ni^2+ (Table 2a) or calcium-free Krebs (n = 4) (Table 2b) in the presence of verapamil markedly reduced or abolished the slow wave. This effect, however, was not immediate. In some cases the upstroke component persisted even at 20 min of perfusion but at a much reduced amplitude, rate of rise and often frequency.

TEA (10 mM) when applied to slow waves without
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<th>Krebs (n=4)</th>
<th>2 mM Ni²⁺ (n=4)</th>
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<td><strong>Resting membrane potential (mV)</strong></td>
<td>-57.5 ± 1.0</td>
<td>-49.5 ± 2.6</td>
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<td><strong>Amplitude (mV)</strong></td>
<td>16.0 ± 0.82</td>
<td>6.5 ± 1.0*</td>
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<td><strong>Duration (msec)</strong></td>
<td>0.59 ± 0.09</td>
<td>0.85 ± 0.06</td>
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<td><strong>Average rate of rise of upstroke (mV sec⁻¹)</strong></td>
<td>95.8 ± 13.4</td>
<td>21.5 ± 8.3*</td>
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<td><strong>Frequency (cpm)</strong></td>
<td>36.8 ± 3.9</td>
<td>28.0 ± 4.0*</td>
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<th>Krebs (n=4)</th>
<th>Ca²⁺-free Krebs (n=4)</th>
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<td><strong>Resting membrane potential (mV)</strong></td>
<td>-57.5 ± 1.5</td>
<td>-48.7 ± 3.6</td>
</tr>
<tr>
<td><strong>Amplitude (mV)</strong></td>
<td>20.3 ± 1.7</td>
<td>4.5 ± 0.96*</td>
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<td><strong>Duration (msec)</strong></td>
<td>0.58 ± 1.7</td>
<td>0.9 ± 0.06*</td>
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<tr>
<td><strong>Average rate of rise of upstroke (mV sec⁻¹)</strong></td>
<td>122.6 ± 20.4</td>
<td>7.0 ± 4.4*</td>
</tr>
<tr>
<td><strong>Frequency</strong></td>
<td>40.2 ± 3.9</td>
<td>31.0 ± 4.5*</td>
</tr>
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*P < 0.05 Significant differences between control and treatment groups (paired t-test).

L-type calcium channel blockade increased the amplitude of the action potentials (Fig. 8, Table 3), but parameters of the slow wave including the frequency and the resting membrane potential were usually not affected, however, in two out of five experiments depolarizations of 4 and 5 mV were observed.

**Characteristics of electrical activities of Sl/Sld mice**

*Action potentials in Sl/Sld mice* Table 1 and Figs 6 and 9 characterize action potentials recorded in Sl/Sld small intestinal muscle preparations. Figure 7 illustrates the recorded variability in the resting membrane potential (−60 to −40 mV) and action potential frequency (0–24 cpm). It also shows that there was a weak linear relationship (r² = 0.45) between the resting membrane potential and action potential frequency, with cells exhibiting the most hyperpolarized resting membrane potential being electrically quiescent. The most characteristic features of the electrical activity as compared with +/+ mice were the complete lack of slow waves and a more depolarized average resting membrane potential. However, many tissues from Sl/Sld mice

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**Figure 8** K⁺ channel blockade with TEA in the small intestine of a +/+ mouse. The first part of top trace shows the electrical activity prior to addition of TEA. At the first arrow TEA (10 mM) was added and at the second removed as indicated. Traces are from a continuous experiment from the same cell, there are 1–2 min lapses between consecutive traces. TEA increases the amplitude of the action potential without affecting the frequency of the slow wave (see Results).  

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<table>
<thead>
<tr>
<th></th>
<th>+/+ (SI/Sp)&lt;sup&gt;d&lt;/sup&gt; (n=5)</th>
<th>SI/Sp&lt;sup&gt;d&lt;/sup&gt; (n=6)</th>
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<tr>
<td></td>
<td>Krebs</td>
<td>10 mm TEA</td>
</tr>
<tr>
<td><strong>Resting membrane potential (mV)</strong></td>
<td><strong>-59.2±3.0</strong></td>
<td><strong>-57.4±3.2</strong></td>
</tr>
<tr>
<td>Amplitude of slow wave (+/+) or slow component (SI/Sp)&lt;sup&gt;d&lt;/sup&gt; (mV)</td>
<td><strong>17.8±2.5</strong></td>
<td><strong>18.4±2.8</strong></td>
</tr>
<tr>
<td>Amplitude of spike (mV)</td>
<td>6.5±0.9</td>
<td>20.0±3.5&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Duration (msec)</td>
<td>0.71±0.05</td>
<td>0.77±0.04</td>
</tr>
<tr>
<td>Average rate of rise of upstroke (+/+) or slow component (SI/Sp)&lt;sup&gt;d&lt;/sup&gt; (mV sec&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td><strong>104.0±15.3</strong></td>
<td><strong>110.0±18.9</strong></td>
</tr>
<tr>
<td>Action potential frequency (cpm)</td>
<td>38.4±1.6</td>
<td>37.2±2.13</td>
</tr>
<tr>
<td>Spike frequency (cps)</td>
<td>4.1±0.6</td>
<td>4.2±0.58</td>
</tr>
<tr>
<td>Spikes per slow wave (+/+) or action potential (SI/Sp)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.0±0.0</td>
<td>2.2±0.2</td>
</tr>
<tr>
<td>Hyperpolarization (mV)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Rate of depolarization rise (mV sec&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>-</td>
<td>-</td>
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<sup>*</sup>P<0.05,  <sup>**</sup>P<0.01 Significant differences between control and TEA (paired t-test).<sup>†</sup>P<0.05,  <sup>‡</sup>P<0.01 Significant difference between +/+ and SI/Sp<sup>d</sup> mice (unpaired t-test).

showed overlapping resting membrane potentials to those of controls (Fig. 6), indicating that alterations in electrical activity were not related to membrane potential differences. The SI/Sp<sup>d</sup> action potentials consisted of a slow prepotential leading to a slow component with superimposed fast spike (s), followed by a hyperpolarization.

Action potentials occurred in different patterns (Figs 9–12). The first type of activity (five out of 20, trace (a) in Fig. 9) consisted of action potentials occurring in bursts of two to five action potentials with a distinguishable quiescent period between the bursts. The average resting membrane potential for this group was -45.4 ± 1.7 mV (range: -50 to -40 mV) and the action potential frequency, 18.7 ± 0.7 cpm (range: 18–24 cpm). The second type of activity (eight out of 20, trace (b) in Fig. 9) showed action potentials without bursting. The average resting membrane potential was -46.4 ± 2.0 mV (range: -54 to -40 mV) and the frequency, 12.9 ± 1.9 cpm (range: 7–20 cpm). The third type (seven out of 20) is represented by trace (c) in Fig. 9, which illustrates a recording from a quiescent period. The average resting membrane potential for this group was -55.0 ± 1.4 mV (range: -60 to -50 mV).

Upon application of K<sup>+</sup>-channel blockers, either 10 mm TEA (n = 3) or 0.5 mm Ba<sup>2+</sup> (n = 7) (Fig. 10), action potentials identical to those recorded in nonquiescent preparations were obtained.

Ba<sup>2+</sup>-induced action potentials were very similar to spontaneously occurring action potentials in SI/Sp<sup>d</sup> preparations (Fig. 6, Table 1). Significant differences were only noted in average resting membrane potential and average rate of rise of the slow component. In addition, SI/Sp<sup>d</sup> tissues in the presence of Ba<sup>2+</sup> showed no relationship between resting membrane potential and action potential frequency (r² = 0.06) and there was less variability in action potential frequency (range: 10–24 cpm) and resting membrane potential (range: -47 to -33 mV) than in nonquiescent SI/Sp<sup>d</sup> tissues.

K<sup>+</sup> channel blockade with TEA A characteristic difference between electrical activities of control and SI/Sp<sup>d</sup> was the response to K<sup>+</sup> channel blockade with TEA. Electrical activity of SI/Sp<sup>d</sup> mice responded to K<sup>+</sup> channel blockade by a marked increase in action potential frequency and/or average rate of rise of the slow component (Table 3). When the action potential frequency exceeded 50 cpm, the high frequency restricted spikes to one per slow oscillation (Fig. 11). The changes appeared independent of effects on the resting membrane potential although in two experiments out of six, the membrane potential decreased by 6 and 8 mV. Similar to control mice, after addition of TEA, the action potential amplitude increased in all experiments. In contrast, in control tissue, TEA did not affect the slow-wave frequency, hence the slow waves restricted the appearance of action potentials to their plateau phase.

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Figure 9 Action potentials in SI/SIP tissues. Traces are from separate experiments, traces (a–c) are without Ba\(^{2+}\); (d–e) in the presence of 0.5 mM Ba\(^{2+}\). SI/SIP small intestine is either quiescent or generates action potentials in a variety of patterns. Action potentials consisting of a slowly developing depolarization leading to a slow component with superimposed spikes and followed by a hyperpolarization occur either in bursts of usually 3–5 action potentials (trace a) with a distinguishable quiescent period in between the bursts or without bursting more or less continuous (traces b, d, and e).

Figure 10 K⁺ channel blockade in quiescent SI/SIP mice. Top traces in (a) and (b) show control traces prior to addition of K⁺ channel blockers. Bottom traces show electrical activity following 3–4 min perfusion with 0.5 mM Ba\(^{2+}\) (a) or with 10 mM TEA (b). Traces (a) and (b) are from separate experiments, each recorded from a continuous experiment. Note different time-scales of both experiments. In quiescent preparations, addition of K⁺ channel blockers (0.5 mM Ba\(^{2+}\) or 10 mM TEA) leads to induction of action potentials.

*L-type Ca\(^{2+}\) channel blockade, Ca\(^{2+}\)-free solution and Ni\(^{2+}\)* In the SI/SIP mice, l-type calcium channel blockade with verapamil (1–10 μM) usually abolished all activity within 15–20 min. An example of l-type Ca\(^{2+}\) channel blockade is included in Fig. 12. The resting membrane potential was unaffected at −41.1 ± 2.0 mV (n = 6) (cf. Table 1). The importance of extracellular Ca\(^{2+}\) was further substantiated by experiments in which either Ca\(^{2+}\)-free solutions or 2 mM Ni\(^{2+}\) were applied. In both cases, the activity was abolished within 3–5 min of perfusion. The resting membrane potential in Ca\(^{2+}\)-free solution was −41.2 ± 2.9 mV (n = 6), not statistically different from the control value of −45.2 ± 2.0 mV (n = 6). Other action potential parameters, prior to application of Ca\(^{2+}\)-free solution, were (n = 6): slow component amplitude, 9.0 ± 2.4 mV, amplitude of spike, 12.8 ± 2.0 mV; slow component duration, 0.76 ± 0.09 sec, average rate of rise of slow component, 44.3 ± 8.9 mV sec\(^{-1}\); action potential frequency, 16.7 ± 4.4 cpm; and spike frequency, 4.5 ± 0.8 cps. The resting membrane potential in the presence of 2 mM Ni\(^{2+}\) was −45.4 ± 1.8 mV (control):
Figure 11 K⁺ channel blockade in S1/SI² mouse. First part of the top trace shows control electrical activity with 0.5 mM Ba²⁺ present throughout the duration of the experiment. At the first arrow 10 mM TEA was added, and removed at the second arrow as indicated. Traces are from a continuous experiment recorded from the same cell, with 0–1 min lapses between consecutive traces. Application of TEA increases amplitude of the action potential (spike component), increases action potential frequency and changes the pattern of the action potential from multiple spikes superimposed on to the slow component to a single spike in this experiment.

Figure 12 L-type Ca²⁺ channel blockade with verapamil in the small intestine of S1/SI² mouse. Top trace shows the control electrical activity with Ba²⁺ (0.5 mM) present throughout the duration of the experiment. Other traces show the electrical activity following 3-, 5-, 10- and 20-min perfusion with 1 μM verapamil as indicated. Traces are from a continuous experiment recorded from the same cell. Verapamil abolishes the action potentials in S1/SI² mice.

45.7 ± 2.1 mV, n = 7), also unaffected. Other parameters, prior to application of Ni²⁺, were: slow component amplitude, 12.8 ± 2.0 mV, amplitude of spike, 10.2 ± 1.7 mV; slow component duration, 0.65 ± 0.08 sec; average rate of rise of slow component, 39.6 ± 6.7 mV sec⁻¹; action potential frequency, 23.9 ± 6.4 cps; spike frequency, 6.4 ± 0.8 cps (n = 7).

DISCUSSION

The major finding was that in S1/SI² mice, there was a complete consistency between lack of slow wave activity, absence of receptor immunoreactivity and absence of interstitial cells of Cajal associated with Auerbach’s plexus of the small intestine (ICC-AP).
The absence of Kit receptor immunoreactivity and ICC-AP in the SI/SI SD mutant proves the dependency of the presence of ICC-AP on the interaction between steel factor and the Kit receptor. This interaction appears to be essential for the development of an ICC-AP network in neonatal mice, days 0–4 pp, but not after this stage, since injected antibodies against the Kit receptor effectively prevent normal development of ICC-AP, when injected days 0–4 pp, but have no recorded effect when injected after day 4 pp.\textsuperscript{6,21} We investigated\textsuperscript{22} the normal development of ICC in neonatal mice, and observed profound changes within 48 h after birth: from a system of few, scattered and apparently not yet connected ICC-AP in uned neonates, to a network of ICC-AP with the adult configuration at 48 h. In contrast to ICC-AP, the adult configuration of the other prominent ICC-network, the ICC-DMP, was recognizable already in the uned neonate.\textsuperscript{23} While the early postnatal development of ICC-AP, but not the later maintenance and survival of the established cell system was controlled by the Kit receptor – steel factor interaction, the ICC-DMP, although Kit receptor positive, were unaffected by the mutations W\textsuperscript{7} and SI.\textsuperscript{12} This indicates a fundamental difference between ICC-AP and ICC-DMP. In support of this view, injection of ACK-2 antibody (against the extracellular domain of the Kit receptor) in newborn mice, while blocking the development of ICC-AP, had no effect on ICC-DMP which remained Kit receptor immunoreactive after three injections and were recognizable by electron microscopy after five injections.\textsuperscript{21} The ICC-DMP was described as altered, since the cytoplasmic electron density was changed.\textsuperscript{21} As an indicator of cellular damage, however, this is a questionable criterion, since in our experience the cytoplasmic electron density of these cells is highly dependent on fixation conditions, perhaps due to a variable degree of contraction of the cells.

The presence of steel factor has been found to suppress apoptosis in haemopoietic cells.\textsuperscript{23} The involvement of a similar mechanism in relation to ICC-AP of the small intestine has not been reported. Ward \textit{et al.}\textsuperscript{12} report the presence of an abnormal Kit-receptor-positive structure in SI/SI SD mice at 5–10 days pp but not in adults, resembling nerve fascicles, but interpreted as abnormal ICC. Further identification, also involving electron microscopy, will be necessary to assess the significance of the finding.

We have, as in our earlier study of W/W SD mice, used methylene blue as a marker for light- and subsequent electron microscopy.\textsuperscript{6} We have found this to be highly reliable, and it is at present the only available marker substance for combined LM and EM. The complete absence of ICC-AP and slow wave activity constitutes strong evidence for the hypothesis that ICC-AP are the intestinal pacemaker cells of the small intestine. It strengthens considerably the evidence provided by studies on W/W SD mice. W/W SD mice lack a functional Kit receptor, have a strongly reduced ICC-AP population (as 5% of control)\textsuperscript{4} and lack slow wave activity. The few ICC-AP present have preserved the typical ultrastructural features, but lack network characteristics. Furthermore, in W/W SD mice a marked increase in fibroblast-like cells (FLC) was observed. Although these cells had the typical fibroblast morphology, it raised the question whether absence of Kit receptor resulted in appearance of a modified ICC with FLC ultrastructure. In SI/SI SD mice, no ICC-AP were identified, nor was there an increase in FLC. It appears that the absence of steel factor completely prevents the development of ICC-AP, whereas the presence of the abnormal Kit receptor as it occurs in W/W SD mice still allows for development of some ICC-AP. It is perhaps likely that studies of a number of mutations at the W and SI loci would reveal even greater variation of viable numbers of ICC-AP.

Our study, and the study by Ward \textit{et al.}\textsuperscript{12} shows the parallel absence of ICC-AP and slow-wave activity in steel-Dickie homozygotes. This does not by itself prove that a causal relationship exists between ICC-AP and generation of slow waves. However, this interpretation is strongly supported by the analysis of morphology, indicating that all other aspects of the normal morphology are preserved in the mutants, including smooth muscle, neural components, other interstitial cell types, such as macrophages and fibroblasts, as well as ICC-DMP. The finding that small-intestinal contractile activity is present in SI/SI SD mice,\textsuperscript{11} has prompted us to analyse the underlying electrical activity in order to understand how such contractile activity develops in the absence of ICC-AP (and slow waves).

The electrical activity of the small intestine of the SI/SI SD mice was generated independently of the ICC-AP and is indistinguishable from that recorded from W/W SD mice. In both cases the tissues are either quiescent or show spontaneous action potential generation without slow waves. In quiescent tissues, activity can be easily evoked through increase in tissue excitability by K channel blockers. It is clear that the presence of action potentials is stimulus dependent and the slow-wave activity is absent at all times. The recorded action potentials have a slowly developing depolarization leading to a slow component with superimposed spikes and followed by a hyperpolarization to below the resting membrane potential. The action potential is
generated through activation of L-type calcium channels. Since isolated single smooth muscle cells generate action potentials similar to those recorded in W/W' or SI/SI' tissues, the electrical activity recorded in these mice reflects intrinsic properties of the smooth muscle cells. While the results from W/W' mice were still slightly ambiguous because of the presence of a limited number of ICC, the current data leave little doubt about the electrical activity in SI/SI' mice representing intrinsic activity of smooth muscle cells, and hence the SI/SI' mouse is an ideal model for the study of muscle function without the influence of the pacemaker. Very often a slowly developing depolarization precedes the action potentials generated in the smooth muscle cells of the SI/SI' small intestine suggesting that the smooth musculature has intrinsic pacemaker activity, which may be similar to other voltage-driven pacemaker systems such as the sinus node tissue in the heart. The action potentials with their slow component and superimposed spikes, which can resemble in appearance slow-wave activity, can easily be distinguished from slow waves pharmacologically. First, the slow component is blocked by L-type calcium channel blockers. Hence it is likely part of the normal action potential generation of smooth muscle. Second, hyperpolarization wipes out action potentials including the slow component but leaves slow waves undisturbed. This indicates that the slow component is evoked by voltage activation of the L-type calcium channel whereas the generation of the slow wave involves the activation of a non-L-type calcium channel in the mouse small intestine as well as in the dog colon. In the dog colon, cAMP and changes in intracellularly stored calcium affected the slow-wave frequency suggesting that intracellular second messengers rather than a voltage change regulate the non-L-type calcium channel. The electrical activity observed in W/W' mice, which is similar to that observed in SI/SI' mice, can generate contractile activity but normal peristaltic activity is not observed. We have preliminary evidence that intestinal transit is made possible through the interaction between the enteric nervous system and the action potentials generated in the smooth musculature. Since both ICC-AP and smooth muscle cells are innervated in mouse small intestine, it is quite likely that the innervation of ICC-AP is primarily related to modulation of slow-wave activity, thereby leaving other nerve/muscle interactions intact in the steel mutants.

The significance of the slow-wave activity in normal tissue becomes clear when comparing Figs 8 and 11. In control tissue, excitation increases action potential generation but the appearance of action potentials remains restricted to the plateau of the slow waves and the slow-wave frequency does not change. Hence, peristaltic activity depending on slow-wave activity will not change either. In contrast, without slow waves, action potential generation increases also upon stimulation, but highly variable patterns of action potentials are generated and consequently highly variable patterns of motor activity.

The intestinal smooth muscle cells of steel and W mutant mice are depolarized relative to controls (this study). A direct influence of ICC-AP on the resting membrane potential of smooth muscle cells is a possibility, the influence could be of a trophic nature, perhaps involving changes in cellular coupling mechanisms. Alternatively, the mechanisms responsible for maintaining the resting membrane potential might be affected by the anemic condition of the mutants. We have observed a change in mitochondrial morphology in intestinal smooth muscle cells of W/W' mice, which is similarly present in SI/SI' mice (Fig. 5). The physiological interpretation of an increase in the number of dumbbell-shaped mitochondria (as seen in cross-sectional profiles) is uncertain, but the changed morphology may well be causally related to a deficiency in oxygen, whether as a sign of damage or indicating a compensatory increase in the dividing rate of mitochondria. The fact that smooth muscle cells are depolarized is probably essential for the occurrence of motor function in SI/SI' mice. If the muscle cells would have been at their normal membrane potential, action potentials would only have occurred in the presence of strongly depolarizing stimuli.

The functional significance of the ICC-DMP is not clear but it has been postulated that the DMP/ICC-DMP associations are involved in the inhibitory regulation of the circular muscle layer. Neural responses to electrical field stimulation were judged normal in SI/SI' mice, with registration of typical excitatory and inhibitory inputs. The present study observed Kit immunoreactivity associated with ICC-DMP in SI/SI' mice, using ACK-2 antibodies against the extracellular domain, previously described by Ward et al. However, in SI/SI' mice no immunoreactivity was observed using Kit antibodies against the intracellular domain, in contrast to control mice. This was a very consistent finding, which might suggest that the development, perhaps conformation, of the Kit receptor may be affected by the absence of the ligand, although without recorded consequences, for ICC-DMP morphology and function.

In summary, the elucidation of the electrical and morphological characteristics of the SI/SI' mouse has given us additional evidence for the designation of the ICC-AP as pacemaker cells. Since our detailed study of
the intestinal morphology has revealed little or no deviation from normal structure of muscle and nerve in addition to the loss of pacemaker cells, the Sl/Spl mutant model appears to be ideally suited for the study of the significance of the lack of slow-wave activity in intestinal motility regulation.

REFERENCES


Chapter Six


Method and contributions

John Malysz performed all electrophysiological experiments, did all analysis of electrophysiological data, and was involved in writing the manuscript and in study design. Dr. Micheal Klüppel (in Dr. Alan Bernstein’s laboratory) performed the in situ *Kit* and *SMMHC* mRNA hibridization, methylene blue staining, and apoptosis experiments. Drs. Jan D. Huizinga and Alan Bernstein initiated the studies, were involved in study design and writing, and coordinated all projects.
Developmental Origin and Kit-Dependent Development of the Interstitial Cells of Cajal in the Mammalian Small Intestine

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ABSTRACT Interstitial cells of Cajal (ICCs) form a network of cells between the external longitudinal and circular muscle layers at the level of the Auerbach's plexus in the mammalian small intestine. These cells express the Kit receptor tyrosine kinase and are essential for intestinal pacemaker activity. W mutant mice carrying structural mutations in the Kit gene lack both the network of ICCs and intestinal pacemaker activity. We were interested in the developmental origin of the cells that make up the network of ICCs. In addition, the specific stages of ICC development that require a functional Kit receptor have not been characterized. We show that ICCs originate from mesenchymal progenitor cells that coexpress both Kit and smooth muscle myosin heavy chain, a marker specific for smooth muscle, during embryogenesis. ICC and longitudinal smooth muscle lineages begin to diverge late in gestation. Embryos homozygous for the regulatory W¹W² (W¹W²) mutation do not express Kit in these mesenchymal progenitor cells. Nevertheless, W¹/W¹ mice display a normal network of ICCs and normal smooth muscle muscles layers at postnatal day 5 (p5). Adult W¹/W¹ mice lack a functional ICC network and intestinal pacemaker activity due to a failure of the ICCs to increase in numbers after p5.

These data suggest a common developmental origin of the ICCs and the longitudinal smooth muscle layers in the mammalian small intestine and show that Kit expression is necessary for the postnatal development and proliferation of ICCs but not for the initial cell lineage decision toward an ICC fate during embryogenesis or for smooth muscle development. Dev. Dyn. 1998;211:60-71.

Key words: interstitial cells of Cajal; developmental origin; W¹; Kit

INTRODUCTION

A century ago, Ramon y Cajal described the specific staining characteristics of the "cellule nerveuse interstitielle" between the external longitudinal muscle (LM) and circular muscle (CM) layers of the intestine at the level of the Auerbach's plexus (Ramon y Cajal, 1893, 1911). For more than 100 years, the function and the developmental origin of these cells remained unclear. Although Ramon y Cajal and others believed that these interstitial cells of Cajal (ICCs) were primitive nerve cells, it has also been suggested that these cells are specialized smooth muscle cells (Faussone-Pellegrini, 1985a), whereas others characterized them as fibroblast-like (for review, see Thuneberg, 1982). Recently, we and others have demonstrated that the cells that form the network of ICCs express the Kit receptor tyrosine kinase (RTK) and, furthermore, that W mutant animals with loss-of-function mutations in Kit (Chabot et al., 1988; Geissler et al., 1988) and Sl mutant animals with loss-of-function mutations in the Kit ligand, Steel factor (Copeland et al., 1990; Huang et al., 1990; Williams et al., 1990; Zsebo et al., 1990), lack both the ICC network and intestinal pacemaker activity (Huizinga et al., 1996; Maeda et al., 1992; Ward et al., 1994). In contrast, the enteric nervous system is not affected in these mutant mice. These observations have demonstrated that the ICCs are necessary for the generation of electrical rhythmicity in intestinal muscle.

ICCs also appear to play an equivalent role in humans: Infantile hypertrophic pyloric stenosis (IHPS), a common pediatric disorder characterized by projectile vomiting, has been suggested to be caused by a lack of coordination between the movements of the pyloric sphincter and the contractions of the stomach (Hayes and Goldenberg, 1957). It has recently been shown that Kit⁺ ICCs are absent in the pylorus of IHPS patients, suggesting that the absence of ICCs is responsible for IHPS (Vanderwinden et al., 1996).

The findings that ICCs are necessary for intestinal pacemaker activity and express RTK raise interesting questions about their developmental origins. The Kit receptor is expressed in diverse cell types, including

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neural crest-derived melanocytes and a subset of sensory neurons of the peripheral nervous system, cells in the central nervous system, primordial germ cells, and hematopoietic cells (Keshet et al., 1991; Motro et al., 1991; Orr-Utregger et al., 1990). Recently, the neural crest has been excluded as the developmental origin of the ICCs in the chick (Lecoin et al., 1996) and in the mouse (Young et al., 1996). Different types of interstitial cells, located either within the CM and LM smooth muscle layers or at the level of the Meissner plexus of the canine intestine, share some morphological and immunohistochemical characteristics with smooth muscle cells (Torihashi et al., 1994). Nevertheless, the different types of interstitial cells in the intestine are morphologically distinct (Thuneberg, 1982; Torihashi et al., 1994), and there is no evidence suggesting that they share a common developmental origin or function. Indeed, the ICCs at the level of Auerbach's plexus are ultrastructurally dissimilar to smooth muscle cells and do not express any of the smooth muscle markers present in other interstitial cells in the gut (Torihashi et al., 1994). In addition, ICCs are morphologically distinct from fibroblasts (Thuneberg, 1982; Torihashi et al., 1994). Moreover, interstitial cells located in the CM layer have a distinct function in mediation of inhibitory neurotransmission (Burns et al., 1996). Thus, ICCs of the Auerbach's plexus in adults are functionally, ultrastructurally, and immunohistochemically distinct from smooth muscle cells as well as other interstitial cells in the small intestine.

Recently, it has been shown that Kit+ cells in the outer layers of the embryonic gut do not coexpress smooth muscle actin or myosin protein (Torihashi et al., 1997). However, those authors did observe cells that coexpressed Kit and Desmin, an intermediate filament protein expressed by smooth muscle cells, in a few cells in the developing LM layer. Thus, they suggested that the few double-positive cells represent smooth muscle progenitor cells, whereas Kit+ cells represent ICC progenitors.

To gain further insight into the developmental origin of the ICCs, we have analyzed the mRNA expression of Kit and the smooth muscle marker smooth muscle myosin heavy chain (SMMHC) in the gastrointestinal tract throughout embryonic development. To elucidate the role of Kit in ICC development, we have studied the effects of two regulatory W mutations, W^57 and W^w^d (W^w^d, Klüppel et al., 1997) on Kit expression, ICC development, and intestinal pacemaker activity.

The results presented here indicate that Kit+ ICCs and SMMHC+ smooth muscle cells develop from a common, mesodermally derived mesenchymal pluripotent precursor that expresses both mRNA markers. Subsequently, we observed a restriction of Kit expression to ICCs and, in parallel, a restriction of SMMHC expression to smooth muscle cells. Thus, these data do not support the hypothesis put forward by Ramon y Cajal and others classifying ICCs as “primitive nerve cells.” In addition, only the W^w^d mutation, but not the W^57 mutation, affects Kit expression, ICC development, and intestinal pacemaking, demonstrating that the genomic inversion in W^w^d DNA (Klüppel et al., 1997) disrupts cis-acting regulatory sequences required for Kit expression in ICCs. Interestingly, Kit expression is not necessary for a lineage decision toward the ICC fate but appears to play a role in postnatal proliferation of ICCs.

RESULTS

Analysis of Kit-Expressing Cells in the Developing Intestine of Wild Type, W^57, and W^w^d Embryos

To gain insight into the developmental origin of the ICCs, we analyzed Kit expression in the developing intestines of wild type, W^57/W^57, and W^w^d/W^w^d mice throughout embryogenesis by RNA in situ hybridization. At embryonic day 10.8 (d10.8) and d11.8, Kit was expressed only in the epithelium of the gut but was not yet evident in the mesenchyme of the developing smooth muscle layers in wild type (Keshet et al., 1991; Motro et al., 1991), W^57, or W^w^d homozygous mutant embryos (data not shown).

At d14.5, both wild type (Fig. 1A,B; Keshet et al., 1991) and W^57/W^57 (Fig. 1C,D) embryos displayed a broad band of strong Kit expression in the developing external muscle layers of the intestine. Occasionally, we observed a restriction of Kit expression reminiscent of later developmental stages (data not shown; see below). Kit expression was completely absent in the external muscle layers of W^w^d/W^w^d embryos at d14.5 (Fig. 1E,F). In contrast, Kit expression in the endothelium was not altered in either W^57 or W^w^d homozygous embryos (Fig. 1A,C,E).

By d16 of embryonic development, Kit expression became restricted to the developing muscle layers in some areas of wild type intestine and already resembled the very localized adult expression pattern of Kit in the ICCs (Fig. 1G, loop 2), whereas other areas still exhibited a broad band of Kit expression (Fig. 1G, loop 1). The pattern of Kit expression was unaffected by the W^57 mutation; in contrast, Kit expression was not observed in the developing muscle layers of W^w^d/W^w^d embryos (data not shown). Interestingly, epithelial expression of Kit was markedly down-regulated and was barely detectable in all three embryos at this stage (Fig. 1G; data not shown).

Two days later, at d18 of embryogenesis, the patterns of Kit expression in wild type (Fig. 1H,I) and W^57/W^57 (data not shown) embryonic gut were restricted to specific groups of cells and single cells in the developing muscle layers in approximately 70% of the intestine, resembling the expression pattern of Kit in the ICCs in the adult. For example, Figure 1J shows two Kit+ cells in the muscularis externa of a d18 wild type embryo. The remaining 30% of the gut at this stage still exhibited a wide distribution of Kit expression in the developing muscle layers (data not shown). Again, there was no expression in W^w^d/W^w^d embryonic gut at this stage (data not shown).
Continuous Restriction of the Numbers of Kit+ Cells During Development of the Outer Muscle Layers

To exclude the possibility that the reduction in Kit expression in the developing muscle layers was due to the selective proliferation of Kit− mesenchymal cells of the muscle layer, we analyzed the numbers of Kit+ and Kit− cells in the developing muscle at different embryonic stages. Only parts of the gut that were sectioned in a round, as opposed to an oval, fashion were used for...
cell counting in order to ensure equivalent comparison between different developmental stages. We analyzed five sections of d14.5 tissue and six sections of d18 tissue from two embryos of each stage. At d14.5, there was an average of 516.2 ± 38.7 cells/section in the muscle layers; all of these cells expressed high levels of Kit (as judged by increased grain density due to hybridization of the radiolabelled Kit probe). In contrast, at d18, the average number of cells in the muscle layer increased to 773.2 ± 48.6, but the number of Kit+ cells decreased to an average of 82.3 ± 13.1 per section. These results demonstrate that the numbers of Kit+ expressing cells in the developing muscle layers decrease during embryogenesis.

Developmental Origin of ICCs

To investigate the developmental origin and lineage relationships of the ICCs, we analyzed both Kit expression and expression of a specific marker of smooth muscle cells (SMMHC; Miano et al., 1994). To determine whether there might be cells that coexpressed both genes, reflecting common cells of origin, we performed single- and double-labeling RNA in situ experiments by using digoxigenin- and radio-labelled SMMHC and Kit cRNA probes on the same sections and on adjacent sections of wild type embryos. In a double-labeling experiment on d14.5 embryos, cells expressing SMMHC were identified by using a digoxigenin-labelled probe and were present in the developing outer muscle layers (muscularis externa) of the gut at d14.5 (Fig. 2A). Strong expression was observed in the inner CM layer, whereas lower but detectably levels of SMMHC expression were present in the outer developing LM layer (Fig. 2A,D,G,J). To confirm expression of SMMHC in this future LM layer, we hybridized sections adjacent to those used in the double-labeling experiment to a radiolabelled SMMHC probe. These experiments confirmed that, whereas the CM layer displayed strong SMMHC expression, the future LM layer expressed lower but detectable levels of SMMHC mRNA at d14.5 of embryogenesis (Fig. 2F,I,L). The differential expression of SMMHC reflects the fact that the inner part of the muscularis externa represents the early-developing CM layer, whereas the outer, lightly labelled area represents the future LM layer, which only differentiates postnatally (Fausson-Pellegrini, 1985b; Torihashi et al., 1997).

At d14.5 of embryogenesis, we observed three main expression patterns for Kit in double-labeling experiments by using a radiolabelled Kit probe. First, in a few areas, Kit was expressed uniformly in both the CM and the future LM layers (Fig. 2B; compare with SMMHC expression in the same section in Fig. 2A).

Second, in most areas, Kit was strongly expressed in the future LM layer, with occasional labelling of cells of the CM layer (Fig. 2E: compare with SMMHC expression in the same section in Fig. 2D and with SMMHC expression in an adjacent section in Fig. 2F; Fig. 2H: compare with SMMHC expression in the same section in Fig. 2G and with SMMHC expression in an adjacent section in Fig. 2I; Fig. 2K: compare with SMMHC expression in the same section in Fig. 2J and with SMMHC expression in an adjacent section in Fig. 2L).

Next, we investigated whether this broad band of Kit expression observed with the radiolabelled probe indicated Kit expression in the majority of cells in the outer layers of the gut. To address this question, we hybridized a sectioned d14.5 embryo with a digoxigenin-labelled Kit probe. Almost all cells of the outer layer were also labelled with this probe, indicating that Kit is expressed in the majority of cells in the developing LM layer (Fig. 2C), as previously demonstrated for kit protein (Torihashi et al., 1997). These results demonstrate coexpression of Kit and SMMHC mRNAs in the area of the future LM layer and in some areas of the CM layer at d14.5.

Third, in a few areas, Kit expression was markedly down-regulated (Fig. 2H,K). By comparing silver grain densities for the Kit and SMMHC radiolabelled probes on adjacent sections, we observed a negative correlation between Kit and SMMHC expression in the future LM layer. In areas of high Kit expression, SMMHC levels were low to intermediate (Fig. 2 H,K), whereas, in areas with reduced Kit expression, SMMHC levels were increased (Fig. 2H,K).

To determine whether Kit and SMMHC continue to be coexpressed at later stages of embryogenesis, we performed double-labeling RNA in situ experiments on d18 embryos. SMMHC was expressed in two distinct layers corresponding to the CM and the developing LM layers (Fig. 2M). The cells located between the CM and LM layers did not express SMMHC (Fig. 2M). Interestingly, Kit was no longer expressed in either the CM or the LM layer but was localized in single cells or groups of cells between the muscle layers (Fig. 2N), suggesting that the ICC and smooth muscle lineages have begun to diverge at this stage in development. Embryos homozygous for the W^7 or W^6 mutations at d14.5 displayed the normal d14.5 SMMHC expression pattern (data not shown), suggesting that the absence of Kit expression in the gut of W^6/W^6 embryos does not interfere with SMMHC expression or smooth muscle development.

Analysis of Kit Expression in Wild Type, W^7, and W^6 Adult Small Intestine

Structural mutations in W/Kit lead to an absence of both the network of ICCs and intestinal pacemaker activity (Huizinga et al., 1995; Ward et al., 1994). To investigate the effects of the two regulatory W mutations, W^7 and W^6, on development of the ICCs in adult small intestine, we performed wholemount RNA in situ experiments on adult small intestine by using a digoxigenin-labelled Kit cRNA probe described previously (Huizinga et al., 1995). After the color reaction, we either separated the external muscle layers from the mucosa and submucosa for better visualization of the ICC network or cross sectioned the tissues. The network of Kit+ ICCs in the separated muscle layers of
wild type small intestine (Fig. 3A) are located between the external LM and CM layers (Fig. 3D), as shown previously (Huizinga et al., 1995; Thuneberg, 1982; Ward et al., 1994). The small intestine of W^{Nt}/W^{Nt} animals displayed an identical pattern of Kit+ ICCs (Fig. 3B) located between the external muscle layers (Fig. 3E). In contrast, we did not observe any Kit+ cells in the separated muscle layers in the small intestines (Fig. 3C) or in the cross-sectioned wholemount tissues (Fig. 3F) of W^{Nt}/W^{Nt} embryos, indicating that Kit expression is either abolished in the ICCs or that the network of Kit-expressing ICCs is absent in W^{Nt}/W^{Nt} animals.

Effects of the W^{Nt} and W^{Ntd} Mutations on ICC Development and Intestinal Pacemaker Activity

To address whether the W^{Nt} or W^{Ntd} mutations affect the development of the ICCs in the small intestine, we stained the gastrointestinal tract of 4-month-old wild type and mutant mice with methylene blue, which, under certain conditions, specifically labels the ICCs
DEVELOPMENT OF INTERSTITIAL CELLS OF CAIL.

Fig. 3. A-F: RNA wholemount in situ analysis of Kit expression in adult small intestine. Shown are isolated muscle layers (A-C) and sections of wholemounted tissues (D-F). A: Wild type small intestine shows a typical network of Kit+ ICCs. D: The ICCs are located between the external CM and LM layers (arrow). The borders of the two muscle layers are indicated by solid lines on the left. B: ICCs in small intestine from W^{+/−}W^{−/−} mice express Kit and form a normal network. E: ICCs from W^{−/−}W^{−/−} mice are located between the two external muscle layers (arrow). C,F: No Kit expression could be detected in the small intestine from W^{−/−}W^{−/−} mice. The arrow (F) shows the expected location of Kit+ ICCs; the solid lines on the left indicate the borders of the muscle layers. G: Supravital methylene blue staining of adult small intestine. G: Wild type small intestine displays the normal network of ICCs, which was also present in the small intestine of W^{+/−}W^{−/−} animals (H). I: The small intestine of W^{−/−}W^{−/−} mice lacks an ICC network; some scattered ICCs are labelled. The photomicrograph in I shows a relatively densely populated area of W^{−/−}W^{−/−} small intestine; other areas are almost completely devoid of ICCs. Scale bar = 100 μm.

Fig. 2. Single- and double-labelling RNA in situ experiments of wild type embryos using a digoxigenin (dig)-labelled and radiolabelled (r) SMMHC and Kit probes in d14.5 (A−L) and d18 (MLN) embryos. A,B: Double-labelling experiment using a dig SMMHC probe and an r Kit probe. The borders of the circular muscle layer (CM; bottom) and the future longitudinal muscle layer (LM; top) are marked by solid lines. A: SMMHC was strongly expressed in the CM layer (arrow), whereas the future LM layer displayed lower levels of SMMHC expression (arrowhead). B: Taking a photomicrograph of the same frame in a different optical plane showed the silver grains indicative of Kit expression. Kit was expressed uniformly in both the CM and the future LM layers. C: Single-labelling experiment using a dig Kit probe demonstrates Kit expression in almost all cells of the outer layers of the muscularis externa. D,E: No Kit expression could be detected in the small intestine from W^{−/−}W^{−/−} mice. E,F: Kit expression became restricted to the future LM layer, whereas some areas of the CM layer retained Kit expression (arrow). F: Section adjacent to the one shown in D and E hybridized to an r SMMHC probe. Similar to D, strong SMMHC expression was found in the CM layer (arrow), whereas a uniform layer of silver grains with a density that was clearly above background was observed in the future LM layer (arrowhead). Double-labelling experiment on two different sections (G,H and J,K) using a dig SMMHC and an r Kit probe. Dashed lines demarcate the inner and outer border of the muscularis externa. In each section, two different loops (labelled 1 and 2) in close proximity are shown (loop 1 and loop 2 in G,H and in J,K are not identical). The location of the number indicates the location of the CM layer. In both G and J, strong SMMHC expression was seen in the CM layer, whereas lower expression was seen in the future LM layer of both loops. H and K show strong expression of Kit in the future LM layer of each loop 2 but decreased Kit expression in each loop 1. Hybridization of adjacent sections to an r SMMHC probe (L for G,H; L for J,K), which resulted in silver grains that were easier to quantitate than the dig color product, demonstrated a negative correlation between Kit and SMMHC expression in the future LM layer. Loop 1 in I and L showed relatively strong SMMHC expression (arrowheads). These tissues had low Kit expression (as shown in H and K). Loop 2 in I and L displayed low (L) to intermediate (I) SMMHC expression in the future LM layers (arrowhead). These tissues had relatively high levels of Kit (as shown in H and K). SMMHC was highly expressed in the CM layers in both I and L (arrows). M,N: Double-labelling of d18 gut. M: SMMHC labels two layers of developing smooth muscle: the inner CM layer and the future LM layer. N: Two Kit+ cells are located between the two muscle layers (silver grains marked by arrowheads) and no longer express SMMHC. Conversely, the SMMHC+ muscle cells no longer express Kit. Scale bar = 30 μm.
(Mikkelsen et al., 1988; Thuneberg, 1982). The small intestines of W<sup>57</sup>/W<sup>57</sup> animals had an apparently normal network of ICCs (Fig. 3H; compare with wild type shown in Fig. 3G), consistent with the normal pattern of Kit expression in these animals (see above). In contrast, the ICC network was absent from W<sup>bd</sup>/W<sup>bd</sup> mice, and only scattered methylene blue<sup>+</sup> cells were detectable (Fig. 3I).

The network of ICCs is essential for intestinal pacemaker activity in the gut, based on the analysis of W structural mutations (Huizinga et al., 1995; Ward et al., 1994). To investigate the effects of the regulatory W<sup>57</sup> and W<sup>bd</sup> mutations on intestinal pacemaker activity, we measured electrical activity of the muscle layers of wild type, W<sup>57</sup>/W<sup>57</sup>, and W<sup>bd</sup>/W<sup>bd</sup> small intestines. Whereas wild type and W<sup>57</sup>/W<sup>57</sup> small intestines displayed the slow wave-type action potentials characteristic of pacemaker activity, the small intestine of W<sup>bd</sup>/W<sup>bd</sup> animals failed to display any slow wave-type action potentials (Fig. 4A). The slow waves exhibited a constant frequency of 35.0 ± 1.6 cpm in control mice and 37.0 ± 4.0 cpm in W<sup>57</sup>/W<sup>57</sup> mice. The resting membrane potentials were somewhat lower in mutant animals (−58.9 ± 1.0 mV in W<sup>57</sup>/W<sup>57</sup>; −49.3 ± 5.2 mV in W<sup>bd</sup>/W<sup>bd</sup>) than in wild type mice (−64.0 ± 4.8 mV, Fig. 4A). The muscle of W<sup>bd</sup>/W<sup>bd</sup> mice was either quiescent (1 of 4) or showed fast, spike-like action potentials at irregular frequency, with an average value of 10.7 ± 2.5 cpm (Fig. 4A), resembling the situation in W/W<sup>+</sup> mice (Huizinga et al., 1995; Ward et al., 1994). The slow-wave component or pacemaker activity of gut smooth muscle is insensitive to L-type calcium channel blockers (Huizinga et al., 1990). In the presence of the blocker D600, the slow-wave component of the action potentials in wild type and W<sup>57</sup>/W<sup>57</sup> mice remained unaltered, whereas the electrical activity of W<sup>bd</sup>/W<sup>bd</sup> mice was completely abolished (data not shown).

These electrical differences in pacemaker activity in the W mutant mice were associated with differences in mechanical contraction patterns (Fig. 4B). Whereas isolated ileum from wild type and W<sup>57</sup>/W<sup>57</sup> animals displayed normal rhythmic, anally propagating contractions, the ilea of W<sup>bd</sup>/W<sup>bd</sup> mice failed to produce these rhythmic contractions and, instead, showed irregular, nondirectional contractions.

**Kit Is Not Required for Embryonic Development of ICCs**

Because mutations at the W locus affect the numbers of ICCs in the adult, it was of interest to determine the step during ICC development that requires a functional Kit receptor. To determine whether Kit is necessary for the lineage determination of multipotential progenitor cells toward an ICC fate during embryogenesis, we first looked for the network of methylene-blue<sup>+</sup> ICCs in the small intestines of W/W<sup>bd</sup> mice after birth. Surprisingly, ICCs were present in normal numbers at postnatal day 5 (p5) in these mutant animals (Fig. 5B; compare with wild type animals shown in Fig. 5A), suggesting that the complete absence of Kit expression in the progenitor cells of the external muscle layers during embryogenesis did not interfere with lineage determination and ICC development up to p5. In contrast, by p15, ICC numbers were significantly reduced in W<sup>bd</sup>/W<sup>bd</sup> animals (Fig. 5C) compared with wild type animals (Fig. 5D). Thus, it appears that Kit is not required for lineage determination of ICCs during embryogenesis but, rather, for postnatal steps in ICC development. Nevertheless, the absence of Kit expression leads to a marked reduction in the density of ICCs in adult animals (see Fig. 3I), suggesting that Kit may be involved in some later developmental processes, such as proliferation or survival of these cells (see Fig. 6).

**Reduction in ICC Density Correlates Negatively With the Increase of the Surface Area of the Small Intestine**

We next asked whether the reduction in density of ICCs in W mutant mice could be explained by the dramatic increase in surface area of the small intestine that occurs in postnatal development. Indeed, the surface of the small intestine of both wild type and W<sup>bd</sup>/W<sup>bd</sup> mice increased approximately 13-fold between p5 and 6 months of age (Table 1). During the same time period, the density of ICCs in W<sup>bd</sup> homozygous animals decreased by approximately the same amount (Table 1). In contrast, the density of ICCs in wild type animals decreased only slightly. By using the values in Table 1, we estimated the numbers of ICCs per small intestine of wild type animals increased approximately 11-fold.
between p5 and 6 months. In contrast, ICC numbers in W^{+/-}W^{+/-} animals appeared not to increase between p5 and p15 and increased less than twofold by 6 months of age. Therefore, in W^{+/-} mutants, the numbers of ICCs throughout the small intestine are not reduced but appear not to increase as dramatically as the numbers in wild type animals.

**ICC Apoptosis in W^{+/-} Mice**

The presence of wild type levels of ICCs in W^{+/-}W^{+/-} newborn mice and the failure of these cells to increase in numbers after birth raised the possibility that the Kit receptor might be required to prevent apoptosis in these cells. In fact, we have shown previously that signaling through RTK prevents p53-dependent programmed cell death in Friend erythroleukemia cells (Abrahamsson et al., 1995). Therefore, we asked whether the reduction in the density of ICCs was associated with increased levels of apoptosis in cells at the levels of the Auerbach's plexus during the first 2 weeks of life. At p4, p8, p9, p10, and p15, the levels of apoptosis according to the results of a Terminal deoxy-Transferase-mediated UTP nick end labeling (TUNEL) assay, were low throughout the tissues examined. More importantly, no differences in the levels of apoptosis between wild type and W^{+/-}W^{+/-} mutant tissues were observed (data not shown).

**DISCUSSION**

The ICCs located between the two external muscle layers of the vertebrate gut at the level of Auerbach's plexus were first described a century ago by Ramon y Cajal (1893, 1911). The developmental origin of this network of cells has been controversial since they were first described. Based largely on morphologic and ultrastructural evidence, they have been considered to be neuronal (Ramon y Cajal, 1893), fibroblast-like, glial-like, or undifferentiated muscle cells (for review, see Thuneberg, 1982). Recently, Lee et al. (1996), by using quail-chick chimeras, demonstrated that the ICCs are not derived from the neural crest, the origin of the enteric nervous system. Lee et al. suggested that
ICCs might be mesodermally derived, originating from splanchnopleural mesenchyme of the lateral plate along with smooth muscle and connective tissue cells.

In this study, we have taken advantage of recent findings by ourselves and others that the \textit{W/Kit} locus is required for normal ICC development (Huizinga et al., 1995; Ward et al., 1994) to investigate the developmental origin and lineage relationship of the ICCs during mouse embryogenesis. We showed that the number of \textit{Kit}+ cells in the muscularis externa becomes continuously restricted between d14.5 and d18 of embryogenesis. Our experiments also suggested that ICCs and smooth muscle cells develop from common, mesodermally derived mesenchymal progenitors, which coexpress both \textit{Kit} and \textit{SMMHC} at midgestation. The three patterns of \textit{Kit} expression observed around d14.5 indicate a dynamic developmental process of ICC and smooth muscle development. First, both the CM layer and the future LM layer coexpress \textit{Kit} and \textit{SMMHC}. Subsequently, \textit{Kit} expression is eliminated in the CM layer, but both markers are still coexpressed in the future LM layer. Thereafter, the divergence of \textit{Kit}+ ICCs and \textit{SMMHC}+ longitudinal smooth muscle cells appears to be initiated, leading to a reduction in the number of \textit{Kit}-expressing cells and to an increase in \textit{SMMHC} expression over the next 4 days of embryonic development. There appear to be spatial differences in the progress through these stages, because we were 

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**TABLE 1. Calculations of Surface Area of the Small Intestine and Numbers of Interstitial Cells of Cajal in Wild Type and \textit{W/Kit} Mice at Different Stages of Postnatal Development$^*$**

<table>
<thead>
<tr>
<th>Age</th>
<th>Surface area of small intestine (cm$^2$)</th>
<th>Numbers of ICC</th>
<th>\textit{W/Kit}/\textit{W/Kit}</th>
</tr>
</thead>
<tbody>
<tr>
<td>p5</td>
<td>$4.75 \pm 0.07$</td>
<td>$526.7 \pm 94.5 \text{ cells/mm}^2$</td>
<td>$531.5 \pm 66.1 \text{ cells/mm}^2$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$2.5 \times 10^9 \text{ cells/ileum}$</td>
<td>$2.5 \times 10^9 \text{ cells/ileum}$</td>
</tr>
<tr>
<td>p15</td>
<td>$8.9 \pm 0.14$</td>
<td>$564.0 \pm 34.2 \text{ cells/mm}^2$</td>
<td>$204.7 \pm 83.7 \text{ cells/mm}^2$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$5 \times 10^9 \text{ cells/ileum}$</td>
<td>$1.8 \times 10^9 \text{ cells/ileum}$</td>
</tr>
<tr>
<td>6 months</td>
<td>$64.4 \pm 2.2$</td>
<td>$442.7 \pm 56.1 \text{ cells/mm}^2$</td>
<td>$64 \pm 44.5 \text{ cell/mm}^2$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$2.8 \times 10^9 \text{ cell/ileum}$</td>
<td>$4.1 \times 10^9 \text{ cell/ileum}$</td>
</tr>
</tbody>
</table>

$^*$The approximately 13-fold increase in small intestine surface area between postnatal day 5 (p5) and 6 months of age is matched by an approximately 11-fold increase in the numbers of interstitial cells of Cajal (ICC) in wild type mice. ICC numbers in \textit{W/Kit}/\textit{W/Kit} (\textit{W/Kit}/\textit{W/Kit}) mice do not increase significantly during the same time period, leading to an approximate eightfold reduction in the density of ICC at the age of 6 months. The average surface area of the small intestines was measured from two mice for each age; numbers of ICC and cell densities were calculated from two mice for each age and genotype.
able to observe all three stages at d14.5. Late in gestation, SMMHC expression is down-regulated in Kit+ ICCs, and, conversely, Kit expression is down-regulated in developing smooth muscle, indicating that both lineages have diverged at that stage. These data provide positive evidence that the putative progenitor cell of ICCs and longitudinal smooth muscle coexpresses both Kit and SMMHC and, thus, that ICCs at the level of the Auerbach's plexus and longitudinal smooth muscle cells derive from a common progenitor cell and might be related cell lineages.

A previous study analyzing the expression of the Kit receptor and markers of the smooth muscle lineage (smooth muscle actin and myosin) was unable to show expression of smooth muscle actin or myosin in the future LM layer before d18 and did not observe coexpression of Kit and these markers in the future LM layers during embryogenesis (Torihashi et al., 1997). Here, we have demonstrated SMMHC mRNA expression in the future LM layer at d14.5 and have shown that Kit and SMMHC are coexpressed during a limited time during embryogenesis. The discrepancy between these studies might be due to the differences in expression assays used: In their study, Torihashi et al. (1997) analyzed protein expression, whereas, in the present study, we analyzed mRNA expression. Alternatively, it is possible that Torihashi et al. might have missed a relatively narrow time window in which Kit and SMMHC are coexpressed. It is possible that mRNA in situ analysis is a more sensitive assay than protein expression assay, a possibility that might explain why Torihashi et al. did not observe the low-to-intermediate levels of SMMHC expression in the future LM layer. Torihashi et al. suggested that smooth muscle cells are derived from a few cells in the future LM layer that coexpress both Kit and Desmin. These authors also suggested that ICCs are derived from Kit+ Desmin- cells in that area. In contrast, our results indicate that the majority of cells in the future LM layer coexpress Kit and SMMHC at d14.5 and are therefore bipotential progenitors that are able to develop along the ICC or smooth muscle lineages. Both this study and that of Torihashi et al. show that ICC lineage determination and divergence between ICCs and smooth muscle occurs between d14.5 (d16 in the Torihashi study) and d18.

The comparative analysis of the effects of two independent W regulatory alleles, W67 and Wwd, on the ICC lineage has also provided further insights into the role of the Kit receptor in ICC development. Although the Wwd mutation abrogates the formation of the network of ICCs in adult homoygous animals, the W67 mutation had no effect on ICC development. Accordingly, only mice homoygous for the Wwd mutation failed to exhibit the electrical and mechanical properties of intestinal pacemaking, whereas W+ mice were normal. Mice homoygous for another regulatory W mutation, Wmut (W9), which is phenotypically and genotypically very similar to the Wwd mutation (Duttlinger et al., 1993; Klüppel et al., 1997), displayed identical defects in ICC development to Wwd mice (data not shown).

These observations are consistent with the loss of Kit expression in the gut mesenchyme of Wwd homozygous embryos and normal Kit expression in the gut mesenchyme during embryogenesis in W67 homozygous embryos. These results demonstrate that the ICC defect in Wwd/Wwd animals results from the down-modulation of Kit expression in the mesenchymal progenitor cells and the developing ICCs. The W67 mutation is the only W mutation known so far that does not affect ICC development in the homozygous state.

Interestingly, the ICCs in Wwd/Wwd mice are present in normal numbers at p5, demonstrating that the Kit receptor is not necessary for either lineage determination of the pluripotent progenitor cell toward an ICC fate or for any subsequent steps of lineage differentiation or proliferation of these cells during embryogenesis. Therefore, activation of the Kit signalling pathway appears to play a permissive role, enabling the mesenchymal progenitor cells that are committed to the ICC lineage to develop further along this pathway. This conclusion is supported by a recent report in which the lacZ marker was inserted into the Kit locus by gene targeting, thus disrupting the coding sequence and creating a Kit null allele. Mice homozygous for this targeted Kit locus died shortly after birth but displayed lacZ-expressing cells in the outer layers of the small intestine at p0, suggesting that Kit is not essential for embryonic development of ICCs (Bernex et al., 1996).

Curiously, the reduction in density of ICCs in the small intestine of Wwd/Wwd animals between p5 and 6 months is not associated with an actual decrease in overall numbers of ICCs. The tissue surface increases about 13-fold during the first 6 months; ICCs of wild type animals expand accordingly, whereas the ICC population in Wwd/Wwd animals is not able to expand. Indeed, the reductions in ICC density in mice homoygous for various W mutations that affect the Kit coding region (Huizinga et al., 1995; Torihashi et al., 1995; Ward et al., 1994) are also consistent with a stagnation in cell numbers.

Without a significant increase in cell numbers, the ICCs in Wwd/Wwd animals are unable to maintain a functional network. This functional ICC network, a requirement for pacemaker activity (Huizinga et al., 1995; Ward et al., 1994; this study), relies on cell-cell contact via long, cellular processes. Thus, if the ICC population cannot maintain a critical density during this increase in the surface area of the small intestine, then cell-cell contacts appear to be disrupted due to the increased space between individual cells, leading to an absence of the synchronized electrical pacemaker activity.

The postnatal plateau in ICC numbers in Wwd/Wwd animals appears to reflect a requirement for the Kit receptor during the postnatal expansion of the ICCs rather than a role for Kit in preventing apoptosis. However, due to the slow postnatal expansion of ICCs, it is possible that apoptosis events are rare at any given
time point and could not be detected in our assays. Figure 6 depicts a model of the development and stages of Kit-dependence of the interstitial cells of cajal.

Previous studies have demonstrated that injection of the Kit-antagonizing Ack2 antibody at p0 and p2, but not before or after, impairs ICC development and intestinal pacemaker activity (Maeda et al., 1992; Turihashi et al., 1995). In contrast, our study indicates effects of the W^d mutation on the density of ICCs only after p5. Therefore, there appears to be a delay of several days between the requirement for Kit and any observable phenotype. In this context, it is of interest to note that stimulation of Kit-expressing melanoblasts with Steel factor at early stages of culture induces subsequent responsiveness to the trophic effects of nerve growth factor (NGF; Langstimm-Sedlak et al., 1996). A similar mechanism could account for the time discrepancy between requirement for Kit and the subsequent reduction in ICC density. According to this model, Kit signaling between p0 and p2 might induce responsiveness of ICCs to proliferative signal(s) by other molecules at later stages in development; in the absence of Kit signaling, this responsiveness would not be induced, and the ICCs would therefore be unable to respond.

Alternatively, the differences between the antibody studies and our results might be due to technical differences. Indeed, effects of Ack2 treatment in adult mice can differ significantly from the effects of W alleles (Galli et al., 1994).

Our results indicate that signaling through the Kit receptor only becomes important after p5, when the ICC network has formed and is located in close proximity to myenteric neurons between the two muscle layers. These neurons express Steel factor (Turihashi et al., 1997); thus, the close proximity between these two cell types might be important for stimulation of ICC proliferation through functional interactions between the membrane-bound form of Steel factor and the Kit receptor. Indeed, S/Kit^dick (S^d) and S^d/Kit^d mutant mice, which lack the membrane-bound form of Steel factor but still express the soluble form of the ligand, display an absence of the ICC network and intestinal pacemaker activity (Huizinga et al., 1995; Ward et al., 1995; Klüppel, unpublished results).

The W^7 and W^d mutations exert tissue- and cell type-specific effects on Kit expression and the development of several cell types, including mast cells and melanocytes (Klüppel et al., 1997). Both the W^7 and the W^d mutations are associated with large genomic rearrangements 5' of the Kit coding sequence. The W^7 chromosome carries an 80-kb deletion 5' to the Kit gene, whereas W^d, like W^d (Klüppel et al., 1997; Nagle et al., 1995), contains a 2.8-Mb inversion 5' of Kit. Both W^7 and W^d affect transcriptional initiation of Kit in a cell-type-specific fashion (Klüppel et al., 1997). Because Kit is normally expressed in ICCs and the W^d mutation affects Kit transcription in ICCs and their mesenchymal progenitor cells, this mutation will be an important tool in the study of transcriptional regulation of Kit in the developing ICCs. The genomic sequences affected by the W^d inversion might provide insights into the interaction of cis-acting elements and transcription factors that control ICC development and establish an ICC-specific program of gene expression.

**EXPERIMENTAL PROCEDURES**

**Mice and Embryos**

C57BL/6J and W^d^7/W^d^7 mice were purchased from Jackson Laboratories (Bar Harbor, ME). C3H, 101, and W^d^101/W^d^101 mice were obtained from the MRC Radiobiology Unit (Chilton, United Kingdom). Embryos were derived from appropriate matings of C57BL/6J or C3H/101 for wild type controls. Mutant embryos were derived from matings of homozygous W^d^7 and W^d^ mice. The noon after vaginal plug was considered day 0.5 p.c.

**RNA in Situ Hybridization**

RNA in situ hybridization on frozen 10-μm sections and wholemount tissues was performed essentially as described previously (Huizinga et al., 1995; Klüppel et al., 1997; Motro et al., 1991). The Kit cRNA probe has been described previously (Motro et al., 1991). Double-labelling in situ hybridization was performed as previously described (Klüppel et al., 1997) by using a digoxigenin-labelled SMMHC cRNA probe (Miano et al., 1994) and a 35S-labelled Kit cRNA probe. Digoxigenin-labelled cells and 35S-labelled cells were in different optical planes due to a parolidon (Fisher Scientific, Fair Lawn, NJ) coating of the slides prior to submersion in emulsion to avoid a color reaction between emulsion and the alkaline-phosphatase color product. Therefore, only two images were taken of the same frame in different optical planes. Sense probes used as controls did not produce any labeling above background.

**Supravital Methylene Blue Staining**

Methylene blue staining of the whole gastrointestinal tract was performed as described previously (Mikkelsen et al., 1988). Briefly, mice were killed by cervical dislocation. The gastrointestinal tract from stomach to rectum was removed, rinsed in phosphate-buffered saline (PBS; without calcium or magnesium), and submerged in 0.7 mM lyssolecithin (Sigma, St. Louis, MO) in PBS for 1 min at room temperature (RT). Tissues were then put in 50 mM methylene blue in fresh Krebs solution in the dark for 40 min at RT. The solution was bubbled with 95% oxygen/5% CO2 or with air throughout the procedure. After the staining, tissues were briefly rinsed in PBS and submerged in a fixative containing picric acid, e.g., Bouin’s fluid. Photomicrographs of the wholemount tissues were taken while the tissues were in the fixative.

**Electrophysiology**

Electrophysiological experiments were performed as described previously (Huizinga et al., 1995).
Apopotosis Assays

We used the Oncor Apoptag-Plus fluorescent apoptosis detection kit (Oncor, Inc., Gaithersburg, MD) on frozen 10-μm sections of small intestines, which were fixed in 4% paraformaldehyde at 4°C for 24 hr followed by soaking in 0.5 M sucrose at 4°C for 24 hr and were embedded in TissueTek (Miles Inc., Elkhart, IN) embedding medium prior to cryosectioning. The assays were performed according to the recommendations of the manufacturer. Counterstaining was performed by using the Hoechst 33258 (Sigma) fluorescent nuclear stain. For a positive control, slides were treated with DNASel (Promega, Madison, WI) prior to the TUNEL procedure; for a negative control, Terminal deoxynucleotidyl Transferase (TdT) was omitted during the procedure.

ACKNOWLEDGMENTS

We thank Dr. Lars Thuneberg for instructions with the methylene blue staining technique and isolation of the external muscles of the small intestine. We thank Ken Harpel for the sectioning of the whole mount tissues and Dr. Joseph Miano for the gift of the SMMHC plasmid. Work in the authors' laboratories is supported by grants from the Medical Research Council of Canada and grant 003313 from the National Cancer Institute of Canada (to A.B.). M.K. was supported by a long-term Government of Canada Award. J.H. is an MRC Scientist.

REFERENCES


Chapter Seven


Methods and contributions

John Malysz performed ~90% of all electrophysiological experiments, did all analysis of electrophysiological data, and was involved in writing the manuscript and in study design. David Richardson and Laura Farraway were involved in the initial pilot study when ideas were first tested, and they carried out ~10% of all electrophysiological experiments. Dr. Marie-Odile Christen provided partial financial support and supplied pinaverium bromide. Dr. Jan D. Huizinga supervised the studies, was involved in study design and writing.
Generation of slow wave type action potentials in the mouse small intestine involves a non-L-type calcium channel

John Malysz, David Richardson, Laura Farraway, Marie-Odile Christen, and Jan D. Hulzinga

Abstract: Intrinsic electrical activities in various isolated segments of the mouse small intestine were recorded (i) to characterize action potential generation and (ii) to obtain a profile on the ion channels involved in initiating the slow wave type action potentials (slow waves). Gradients in slow wave frequency, resting membrane potential, and occurrence of spiking activity were found, with the proximal intestine exhibiting the highest frequency, the most hyperpolarized cell membrane, and the greatest occurrence of spikes. The slow waves were only partially sensitive to L-type calcium channel blockers. Nifedipine, verapamil, and pinaverium bromide abolished spikes that occurred on the plateau phase of the slow waves in all tissues. The activity that remained in the presence of L-type calcium channel blockers, the upstroke potential, retained a similar amplitude to the original slow wave and was of identical frequency. The upstroke potential was not sensitive to a reduction in extracellular chloride or to the sodium channel blockers tetrodotoxin and mexiletine. Abolishment of the Na⁺ gradient by removal of 120 mM extracellular Na⁺ reduced the upstroke potential frequency by 13–18% and its amplitude by 50–70% in the ileum. The amplitude was similarly reduced by Ni²⁺ (up to 5 mM), and by flufenamic acid (100 μM), a nonspecific cation and chloride channel blocker. Gadolinium, a nonspecific blocker of cation and stretch-activated channels, had no effect. Throughout these pharmacological manipulations, a robust oscillation remained at 5–10 mV. This oscillation likely reflects pacemaker activity. It was rapidly abolished by removal of extracellular calcium but not affected by L-type calcium channel blockers. In summary, the mouse small intestine has been established as a model for research into slow wave generation and electrical pacemaker activity. The upstroke part of the slow wave has two components, the pacemaker component involves a non-L-type calcium channel.

Key words: slow wave, pacemaker, calcium channel, pinaverium, smooth muscle.

Résumé : On a enregistré les activités électriques intrinsèques dans divers segments isolés de l'intestin grêle de souris (i) pour caractériser la formation des potentiels d'action et (ii) pour obtenir un portrait des canaux ioniques participant au déclenchement des potentiels d'action de type ondes lentes. On a observé des gradients dans la fréquence des ondes lentes, le potentiel de repos de la membrane et l'activité des points d'action ; l'intestin proximal avait la fréquence la plus élevée, la membrane cellulaire la plus hyperpolarisée et l'occurrence de points d'action la plus importante. Les ondes lentes n'ont été que partiellement sensibles aux bloqueurs de canaux calciques de type L. Dans tous les tissus, la nifédipine, le vérapamil et le bromure de pinaverium ont éliminé les points d'action qui sont apparus pendant la phase de plateau des ondes lentes. L'activité qui a persisté en présence des bloqueurs de canaux calciques de type L, c'est-à-dire le potentiel ascendant, a maintenu une amplitude similaire à l'onde lente originale en plus d'avoir une fréquence identique. Le potentiel ascendant a été insensible à une réduction de chlorure extracellulaire et aux bloqueurs de canaux sodiques, tetrodotoxine et mexiletine. Dans l'iléon, l'élimination du gradient Na⁺ par le retrait de 120 mM de Na⁺ extracellulaire a réduit la fréquence du potentiel ascendant de 13–18%, et son amplitude de 50–70%. L'amplitude a été réduite de façon similaire par le Ni²⁺ (jusqu'à 5 mM) et par l'acide flufenamique (100 μM), un bloqueur non spécifique des canaux chlorure et cationiques. Le gadolinium, un bloqueur non spécifique des canaux sensibles à l'étrier et des canaux cationiques, n'a eu aucun effet. Pendant tous ces essais pharmacologiques, une forte oscillation s'est maintenue entre 5 et 10 mV. Cette

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1 Author for correspondence.

oscillation reflète probablement l'activité « pacemaker »; elle a été rapidement éliminée par le retrait de calcium extracellulaire, mais n'a pas été affectée par les bloqueurs de canaux calciques de type L. En résumé, l'intestin grêle de souris est un modèle pour la recherche dans la production d'ondes lentes et l'activité « pacemaker » électrique. La partie ascendante de l'onde lente a deux composantes: la composante « pacemaker » se met en jeu un canal calcique qui n'est pas de type L.

Mots clés : onde lente, « pacemaker », canal calcique, pinavérium, muscle lisse.

[Intraduit par la Rédaction]

Introduction

Our knowledge about pacemaker activity in the gastrointestinal tract is advancing along several fronts. It has become clear that pacemaker activity is generated in specialized areas of the gut organs with interstitial cells of Cajal (ICC) playing an essential role (Thuneberg 1982; Fausson-Pellegrini 1992; review; Thuneberg et al. 1995; Daniel and Berezin 1992; Christensen et al. 1992; Sanders et al. 1991; Publicover 1995). There is overwhelming structural and electrophysiological evidence for this in the mouse small intestine (Huizinga et al. 1995; Thuneberg 1989; Maeda et al. 1992; Ward et al. 1994; Fausson-Pellegrini 1985). The mouse is therefore an ideal model for the study of pacemaker activity. One of the key strategies in elucidating pacemaker activity is to characterize the ion channels involved in its generation.

Taking into account all species and tissues, no firm evidence exists about specific ion channels involved in generating pacemaker currents. In the canine colon, it has been proposed that a non-L-type calcium channel is involved in initiating slow wave type action potentials (Huizinga et al. 1991b; Liu et al. 1995a; Mollemann et al. 1995), although others hold it as possible that L-type calcium channels (Triggles 1990) may be primarily responsible for the slow wave (Ward and Sanders 1992a; Langton et al. 1989). Pacemaker currents in the cat colon probably also primarily involve calcium (Snapc and Tan 1985). In the small intestine, evidence points to the involvement of calcium and sodium conductances. In the rabbit small intestine (El-Sharkawy and Daniel 1975) the slow wave is abolished by the removal of either Na⁺ or Ca²⁺ ions from the extracellular medium. Consistent with findings in the rabbit small intestine, Liu et al. (1969) found that the slow waves in the cat small intestine were abolished by the removal of extracellular Na⁺. They also found that the omission of Ca²⁺ causes a decrease in the rate of rise of the slow waves. These results were confirmed in a later study by Dahms et al. (1987). In both studies the conclusion was that the upstroke potential was the result of an influx of Na⁺. Since no known Na⁺ channel found in smooth muscle is a likely candidate, the involvement of a sodium conductance in slow wave generation warrants further study.

Because of the uncertainties surrounding calcium and sodium involvement in the initiation of the slow wave type action potential in the small intestine, we carry out patch clamp studies on single cells (Mollemann et al. 1993; Lee et al. 1993a) and intracellular recordings in tissue to study action potentials from the mouse small intestine. This study is the first to investigate the ionic basis of electrical rhythmicity in the mouse small intestine. It generates a hypothesis on the nature of the ion channels responsible for pacemaking activity in the mouse small intestine.

Methods

Tissue preparation

Mice of either sex (CD1, Charles River Laboratories, St-Constant, Que.) were sacrificed by cervical dislocation. The small intestine was then exposed by a midline abdominal incision, and a segment of the small intestine was removed. Duodenal sections, 3–5 cm, were removed starting 1 cm from the pylorus. The distal end of the ileal segments were removed 1–2 cm proximal to the cecum. The segments were placed in a dissecting dish filled with oxygenated (95% O₂ – 5% CO₂) Krebs solution.

Preparation and recording of the electrical activities

The preparations were opened flat and the contents were removed. The mucosa was then carefully removed by sharp dissection. Muscle strips, 15 mm long, were prepared by cutting them parallel to the longitudinal muscle bundles; the tissue was 0.5–0.8 cm wide with a length of 1.0–2.0 cm. The muscle was spread flat over the Sylgard (Dow Corning Corporation, Midland, Mich.) bottom of a transfer holder. The preparation was mounted by carefully putting pins forming a 3 x 3 mm square. The tissue was then placed in an Abe-Tomita-type bath (Abe and Tomita 1968) with a 6-mL capacity. The preparation was perfused with Krebs solution at a constant rate (5.0 mL/min) at 36.0–37.0°C.

Intracellular recordings were made by microelectrodes with 40–100 MΩ tip resistance. Microelectrodes were prepared from 1.2 mm outside diameter glass capillaries (WPI Inc., Sarasota, Fla.) and filled with 3 M KC1. A micro-electrode was inserted into a microelectrode holder (WPI M700P) that connected to an electrometer (WPI M-707A), which is a high impedance probe. The output of the electrometer was displayed on an oscilloscope (1425; Gould, Cleveland, Ohio) and recorded on a Gould ink-writing recorder (2200S).

Microelectrodes were driven perpendicularly into the tissue in steps of 1 μm, with or without the use of a Burleigh 7010 inchworm controller (Burleigh Instruments Inc., Fishers, N.Y.). At each step a short (~25 ms) voltage oscillation was derived by the preamplifier to impale a cell.

Preparation and recording of mechanical activities

Whole ileal segments, 5 mm in length, were cut, and the contents were carefully removed. Small pieces of plastic were inserted into both ends and secured in place using silk thread. Segments were placed in a 20-mL organ bath chamber filled with continuously oxygenated Krebs solution, at 37.0 ± 0.5°C, and then mounted to force transducers. The segments were stretched to obtain optimal spontaneous contractile
Table 1. Compositions of solutions (in mM).

<table>
<thead>
<tr>
<th></th>
<th>Normal Krebs</th>
<th>Ca(^{2+})-free Krebs</th>
<th>Low Cl(^-) (isethionate)</th>
<th>Low Na(^+) (choline)*</th>
<th>Low Na(^+) (glucamine)</th>
<th>Ca(^{2+})-free and low Na</th>
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<tr>
<td>NaCl</td>
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<td>120.3</td>
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<td>-</td>
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<td>1.2</td>
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<td>1.2</td>
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<tr>
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<td>120.3</td>
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<td>-</td>
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<td>N-Methyl-d-glucamine</td>
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<td>-</td>
<td>-</td>
<td>120.3</td>
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<tr>
<td>Choline chloride</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>120.3</td>
<td>-</td>
<td>-</td>
</tr>
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<td>21.2</td>
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<td>5.9</td>
<td>5.9</td>
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</tr>
<tr>
<td>Ca(^{2+})</td>
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<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
<td>0</td>
</tr>
<tr>
<td>Cl(^-)</td>
<td>133.6</td>
<td>128.6</td>
<td>13.3</td>
<td>133.6</td>
<td>20.4*</td>
<td>20.4*</td>
</tr>
</tbody>
</table>

*Experiments with choline chloride were carried out in the presence of atropine. Atropine did not affect the upstroke potential.

The pH of these solutions was adjusted with HCl, which brought the [Cl\(^-\)] to the values stated.

Fig. 1. Verapamil abolishes spiking and contractile activities in the ileum. (a) Sections from a continuous experiment in the ileum. There are 3- to 5-min lapses between traces. The top trace shows the activity recorded in Krebs solution; at the arrow 2 \(\mu\)M verapamil was applied. (b) A separate mechanical study illustrating abolishment of contractile activity with verapamil (2 \(\mu\)M) added at the arrow. (c) Expanded traces of experiment in Fig. 1a. They characterize spiking activity and its blockade by 2 \(\mu\)M verapamil.

(a) 120 mV ↓

-60 mV

(b) 5 mN ↓

1 min

(c) 20 mV

-60 mV

6 s

Data were obtained usually from the same cells, before and in the presence of a drug or after ion or solution substitution. A difference was considered to be significant at \(p < 0.05\). Data are expressed as means ± SEM. Paired and unpaired Student's \(t\) tests were employed for comparisons of electrical and mechanical activities whenever appropriate.

The animals were cared for in accordance with the principles and guidelines of the Canadian Council on Animal Care.

Solutions and drugs
The composition of the Krebs solution was (in mM): NaCl, 120.3; KCl, 5.9; CaCl\(_2\), 2.5; MgCl\(_2\), 1.2; NaHCO\(_3\), 20.0; Na\(_2\)H\(_2\)PO\(_4\), 1.2; and glucose, 11.5. The pH was 7.30–7.35. Compositions of other solutions are summarized in Table 1. Other chemicals used were verapamil (Sigma Chemical Corp., St. Louis, Mo.), NiCl\(_2\) (Sigma), tetrodotoxin (Sigma), mepeteline HCl (Boehringer Ingelheim, Germany), pinaverium bromide (Solvay Pharma, France), nifedipine (Sigma), gadolinium chloride (Sigma), flufenamic acid (Sigma), and atropine (Sigma).

Results
The musculature of the mouse small intestine generated spontaneous slow wave type action potentials (slow waves) at extremely regular frequency in all preparations studied.
(Fig. 1; Table 2). In the duodenum (n = 13), from a resting membrane potential of ~63.0 mV (Table 2), the amplitude of the slow wave type action potentials reached 30.4 mV, including spikes that occurred superimposed on the plateau phase of the slow waves. The slow wave frequency was 44.8 cycles per minute (cpm). The slow wave type action potentials were recorded throughout the small intestine, but quantitative changes were noted gradually when recordings were made more and more distally. In the ileum, the frequency of the slow wave type action potentials was reduced to 36.0 cpm, and the resting membrane potential was more depolarized to ~57.4 mV. The frequency in the distal ileum was as low as 24 cpm in some preparations. In the ileum, the slow wave amplitude was 30.1 mV, including spikes. Other parameters are given in Table 2.

L-type calcium channel blockers
Blockade of L-type calcium channels by nifedipine (1 μM), verapamil (1–5 μM), or pinaverium bromide (5 μM) abolished spiking activity superimposed on the slow wave (Table 2). Other slow wave parameters were not significantly affected, although in less than 10% of cases the frequency slightly decreased by less than 25%. In the presence of L-type calcium channel blockers, a very robust part of the slow wave remained, defined as the upstroke potential consistent with work on other tissues (Huizinga et al. 1991b). All subsequent experiments on electrical activity were carried out in the presence of verapamil, 2 μM.

A separate study on the mechanical activity of segments of small intestine revealed that verapamil abolished contractions (Fig. 2b). Prior to addition of verapamil, the average frequency and amplitude of contractions were 33.8 ± 1.9 contractions per minute and 3.4 ± 4.9 mN (n = 6), respectively.

Role of Cl⁻ ions
The upstroke potential in the mouse small intestine was not sensitive to reduction of extracellular Cl⁻ (to 17% of its original concentration) substituted by iodothionate (Fig. 3). In three of six experiments, the treatment slightly reduced the slow wave frequency.

Role of Na⁺ ions
Because current hypotheses propose that a sodium conductance is responsible for the generation of slow waves in the small intestine (El-Sharkawy and Daniel 1975; Liu et al. 1969), we investigated the sensitivity of the upstroke potential to abolishment of the Na⁺ gradient, which effectively excludes passive sodium influx. In the duodenum and ileum, low Na⁺ solutions decreased amplitude, frequency, and rate of rise of the upstroke potentials. Low sodium solutions reduced the upstroke potential amplitude by 60% in the duodenum and by 50–70% in the ileum and the frequency by 52% in duodenum and by 13–18% in the ileum (Figs. 2, 3, and 4). The slow wave duration increased from 0.6 ± 0.1 to 0.9 ± 0.1 s (n = 5, p < 0.05) in the duodenum, and from 0.8 ± 0.1 to 1.0 ± 0.1 s (n = 7, p < 0.05) in the ileum. In the presence of these low sodium solutions an upstroke potential component of 4–10 mV, always remained (Figs. 2, 3, and 4).

Because of the Na⁺ sensitivity of the upstroke potential, it was of interest to study the effects of the Na⁺ channel

<table>
<thead>
<tr>
<th>Table 2. Comparison of slow wave type action potential characteristics in duodenum and ileum and effects of L-type Ca²⁺ channel blockade.</th>
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<tbody>
<tr>
<td>Parameter</td>
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<td>-------------------------------------------------</td>
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<tr>
<td>Amplitude (mV)</td>
</tr>
<tr>
<td>Frequency (cpm)</td>
</tr>
<tr>
<td>Resting membrane potential (mV)</td>
</tr>
<tr>
<td>Duration of slow wave (s)</td>
</tr>
</tbody>
</table>

Note: Significant differences (paired t-test) are noted between duodenum and ileum at p < 0.05 (%) and p < 0.001 (%).
Fig. 2. Effects of various experimental conditions on the electrical activity in the ileum in the presence of verapamil. Significant differences (paired t test) are shown at \( p < 0.05 \) (*) and \( p < 0.01 \) (**). The \( n \) values for low Na\(^+\) (glucamine), low Na\(^+\) (choline), Ca\(^{2+}\)-free, low Na\(^+\) and Ca\(^{2+}\)-free, 2 mM Ni\(^{2+}\), and low Cl\(^-\) (isethionate) are 7, 5, 7, 4, 7, and 6, respectively. Closed bars, control; cross-hatched bars, experimental condition.

Resting Membrane Potential (mV)

Amplitude (mV)

Rate of Rise (mV/s)

Frequency (cpm)

blockers, tetrodotoxin (TTX) and mexiletine. TTX-sensitive Na\(^+\) channels have been observed in the rat ileum and other smooth muscle (Sturek and Hermensreyer 1986; Molleman et al. 1991; Smirnov et al. 1992; Xiong et al. 1993), while mexiletine effectively blocked the inward Na\(^+\) current responsible for the depolarization phase of the action potential in cardiac muscle preparations (Yatani and Akaike 1985; Hering et al. 1983). In our study, both TTX (0.3 \( \mu \)M, \( n = 4 \)) and mexiletine (10 \( \mu \)M, \( n = 5 \)) had no effect on the upstroke potentials, in both the duodenum and ileum (Table 3).

Role of Ca\(^{2+}\) ions

The upstroke potentials in the mouse small intestine were sensitive to removal of extracellular Ca\(^{2+}\) (Fig. 4). However, there was a large variability in the time it took for Ca\(^{2+}\) removal to take full effect. In some cases, the upstroke potentials disappeared within 5 min of superfusion, in other preparations they remained even after 20 min albeit at a reduced amplitude, frequency, and rate of rise. Addition of EGTA (5 mM) (data not shown) to zero Ca\(^{2+}\) solutions increased the rate of reduction in upstroke potential amplitude, but still in some preparations, activity could be observed after 15 min of perfusion (Figs. 2 and 3). Addition of EGTA usually resulted in slight depolarization.

When preparations superfused by a low Na\(^+\) — glucamine solution were subsequently subjected to a Ca\(^{2+}\)-free low Na\(^+\) — glucamine solution, the oscillatory activity was abolished immediately without changes in resting membrane potential (Fig. 4c). Also, simultaneous removal of extracellular Ca\(^{2+}\) (plus 5 mM EGTA) and reduction of extracellular Na\(^+\) (glucamine solution) abolished the upstroke potential within 1–5 min of perfusion (Figs. 2, 3, and 4d).

Effects of Ni\(^{2+}\), flueneamic acid, and gadolinium

Since both Na\(^+\) and Ca\(^{2+}\) ions appeared to be involved in the generation of the upstroke potential, the possibility was considered that a nonselective cation channel was involved.

Ni\(^{2+}\) blocks many nonspecific cation channels (Inoue 1991; Kim and Woodruff 1991) and its effect was studied in both duodenal and ileal preparations (Figs. 2, 3, and 5). Ni\(^{2+}\) had its most significant effect on the amplitude and rate of rise of the upstroke potentials. The amplitude of the upstroke potentials was reduced to 5–10 mV in the ileum (Fig. 2) and 3–7 mV in the duodenum (Fig. 3). The rate of rise of the upstroke was dramatically reduced in both ileum and duodenum (Figs. 2 and 3). Ni\(^{2+}\) reduced the frequency (Figs. 2 and 3) and increased the duration of the upstroke potentials in the ileum from 0.8 ± 0.1 to 1.1 ± 0.15 s \( (p < 0.05; n = 7) \) and in the duodenum from 0.6 ± 0.1 to 1.1 ± 0.2 s \( (p < 0.01; n = 4) \). The effect of Ni\(^{2+}\) was similar to that of removal of extracellular Na\(^+\) (Figs. 2, 3, and 5). The effects of Ni\(^{2+}\) occurred without significant changes in the resting membrane potential within the first 10 min of perfusion; however, in three of seven experiments in the ileum and three of four in the duodenum, the resting membrane potential decreased after 10 min (Figs. 2 and 3).
Fig. 3. Effects of various experimental conditions on the electrical activity in the duodenum in the presence of verapamil. Significant differences (paired t test) are shown at \( p < 0.05 \) (*) and \( p < 0.01 \) (**). The \( n \) values for low Na\(^+\) (glucamine), Ca\(^{2+}\)-free plus 5 mM EGTA, low Na\(^+\) and Ca\(^{2+}\)-free, and 2 mM Ni\(^{2+}\) are 5, 6, 5, and 4, respectively. Closed bars, control; hatched bars, experimental condition.

<table>
<thead>
<tr>
<th>Low Na(^+) (Glucamine) 10 min</th>
<th>Low Na(^+) (Glucamine) 20 min</th>
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<tr>
<td>-55.3±2.5</td>
<td>-55.3±2.5</td>
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<table>
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<tr>
<th>Ca(^{2+})-Free + EGTA 10 min</th>
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<td>-60.8±3.2</td>
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<th>Low Na(^+) &amp; Ca(^{2+})-Free 10 min</th>
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<tr>
<td>2 mM Ni(^{2+}) 10 min</td>
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<tr>
<td>2 mM Ni(^{2+}) 20 min</td>
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<td>-61.0±3.1</td>
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<th>Rate of Rise (mV/s)</th>
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<td>80</td>
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Table 3. Effects of various channel blockers on the upstroke potential in ileum in presence of verapamil.

<table>
<thead>
<tr>
<th></th>
<th>Resting membrane potential (mV)</th>
<th>Amplitude (mV)</th>
<th>Frequency (cpm)</th>
<th>Duration (s)</th>
<th>Average rate of rise (mV/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 4)</td>
<td>-65.3±2.5</td>
<td>15.5±2.1</td>
<td>37.5±2.9</td>
<td>0.70±0.05</td>
<td>72.5±10.5</td>
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<tr>
<td>TTX, 15 min, 3 × 10(^{-7}) M (n = 4)</td>
<td>-65.3±2.5</td>
<td>15.8±1.7</td>
<td>37.5±2.9</td>
<td>0.75±0.05</td>
<td>75.0±9.6</td>
</tr>
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Control (n = 5)

| Mexiletine 10 min, 10 \(^{-3}\) M (n = 5) | -60.8±2.9 |
| 20 min, 10 \(^{-3}\) M (n = 5)               | -61.0±3.1 |

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| Control (n = 4) | -63.0±3.7 |
| Gadolinium, 10 min, 100 \(\mu\)M (n = 4) | -62.5±3.9 |

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| Control (n = 4) | -61.3±2.9 |
| Flufenamic acid, 10 min, 10 \(\mu\)M (n = 4) | -61.5±2.8 |

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| Control (n = 6) | -57.3±2.3 |
| Flufenamic acid, 10 min, 100 \(\mu\)M (n = 6) | -57.3±2.3 |

```

*\(p < 0.05\).

**\(p < 0.01\).

Importantly, even at a Ni\(^{2+}\) concentration of 5 mM, oscillatory activity at 4 mV remained, at a slightly reduced frequency (Fig. 5d).

Flufenamic acid is a nonspecific cation channel blocker (Siemers and Gogolen 1993a (review), 1993b; Chen et al. 1993), albeit rather nonspecific since it also blocks Cl\(^-\) channels and opens certain K channels (Farrugia et al. 1993). Two concentrations were tested, 10 and 100 \(\mu\)M; the lower
Fig. 4. Effects of reduction of extracellular Na⁺ and removal of extracellular Ca²⁺ on electrical activity in the presence of verapamil. (a) Effect of removal of Ca²⁺ (at arrow) from the perfusing medium in the ileum. Traces are from a continuous recording. There are 3- to 5-min lapses between consecutive traces. (b) Effect of reduction of Na⁺ and substitution with N-methyl-D-glucamine (at arrow) in the ileum. A stable component remains at a reduced amplitude, a reduced rate of rise, and a slightly reduced frequency. Traces are from a continuous recording. There are 3- to 5-min lapses between consecutive traces. The first trace in Fig. 4c shows oscillatory activity in low Na⁺ (glucamine). The activity is completely abolished when Ca²⁺ is removed by superfusion with zero Ca²⁺, low Na⁺ (glucamine), EGTA (5 mM). The traces are from a continuous experiment and there are 1- to 2-min lapses between tracings. (d) Effect of simultaneous reduction of Na⁺ and removal of Ca²⁺ (plus 5 mM EGTA) in the duodenum. At the arrow, the solution was changed from control to low Na⁺ (glucamine) and Ca²⁺-free. The electrical activity quickly abolished.

concentration had no effect, while 100 μM reduced the amplitude and the rate of rise of the upstroke potentials with no effect on the frequency (Fig. 5, Table 3). The effects were independent from changes in the resting membrane potential, although in two of six experiments, the membrane potential decreased.

Gadolinium, in the micromolar range, blocks stretch-activated ion channels (Wellner and Isenberg 1994) as well as nonspecific cation channels (Nonaka et al. 1995; Fernando and Barratt 1994) and T-type calcium channels (Mlinar and Eysart 1993). Gadolinium (100 μM) had no effect on the upstroke potential.

Discussion

Addition of L-type calcium channel blockers had a profound effect on the activity of the mouse small intestine, rapidly abolishing spiking activity and contractile activity. However, in the presence of these blockers a substantial part of the slow wave, the upstroke potential, remained at unchanged frequency.

The upstroke potential consisted of two components. One component was abolished by removal of extracellular Na⁺ or Ca²⁺. This component could be associated with a nonspecific cation channel since Ni²⁺ (2-5 mM), as well as flufenamic acid (100 μM), abolished it. Once the Na⁺-sensitive component was removed, a second component remained, at 5-10 mV amplitude and 27-32 cpm frequency, very sensitive to removal of extracellular Ca²⁺ and also sensitive to Ni²⁺. This robust oscillation will encompass the pacemaker potential. The most likely candidate for the ion channel generating the pacemaker potential is a non-L-type calcium channel. We have not found a blocker for this Ca²⁺-sensitive component, although it is partially inhibited by Ni²⁺. Nonselective blockers of nonspecific cation channels, flufenamic acid and gadolinium, had no effect on the pacemaker potential. The identity of channel(s) associated with the pacemaker potential remains to be elucidated. There are remarkable similarities with the pacemaker potential in colon smooth muscle (Ward and Sanders 1992a; Huizinga et al. 1991b). In the dog colon, the upstroke potential is insensitive to voltage changes (Huizinga et al. 1991a) but very sensitive to changes in intracellular calcium (Liu et al. 1995b) and cAMP (Huizinga et al. 1991a). Preliminary data on the mouse small intestine also show marked effects of cAMP and cyclopiazonic acid (CPA) on the upstroke frequency (J.D. Huizinga, D. Richardson, and J. Malyasz, unpublished observations). This suggests that also in the small intestine the pacemaker is linked to intracellular biochemical processes.

A two-component model for the characterization of the upstroke potential was earlier proposed by Tomita (1981). He derived most of his data from the guinea pig stomach. Tomita also came to the conclusion that the pacemaker component is a voltage-insensitive part of the slow wave. He sug-
Fig. 5. Effects of Ni²⁺ and flueneamic acid on electrical activity in the mouse small intestine in presence of verapamil. (a) Effect of 2 mM Ni²⁺ (added at the arrow) on the upstroke potential in the ileum. Ni²⁺ partly inhibits the upstroke. Traces are from a continuous experiment; there are 2- to 5-min lapses between traces. (b) Effect of 2 mM Ni²⁺ on the Na⁺-insensitive component in the ileum from a continuous experiment. Ni²⁺ was added at the arrow to the perfusing solution containing gluclidean Krebs solution and verapamil. Ni²⁺ further blocks the remaining (Ca²⁺) component. There are 1- to 2-min lapses between traces. (c) Effect of flueneamic acid on the electrical activity in the duodenum. There are 2- to 5-min lapses between traces; traces are from a continuous experiment. (d) Effect of 5 mM Ni²⁺, added at the arrow, on the upstroke in the duodenum.

(a)  
-52 mV
-52 mV
-82 mV

(b)  
-82 mV

(c)  
-64 mV

(d)  
20 mV  
15 s
-64 mV

gested that an ion exchanger might play a role. An ion channel activated by an intracellular component would also fit his model.

In the rabbit small intestine, El-Sharkawy and Daniel (1979) found that slow wave activity was abolished within 20 min of removing extracellular Na⁺. They also found slow wave activity to be sensitive to omission of Ca²⁺. They concluded that the upstroke potential was generated by an inward Na⁺ current that may be dependent on the presence of external Ca²⁺. In the cat small intestine (Liu et al. 1969), it was found that removal of extracellular Na⁺ abolished slow wave activity in 40–60 min. These authors also found that omission of Ca²⁺ had effects on the slow wave by decreasing the rate of rise. These studies logically concluded that a sodium conductance was likely involved in slow wave generation. However, no Na⁺ channel in smooth muscle has been identified that would be a candidate. In the rat ileum and other smooth muscles, a TTX-sensitive sodium channel has been identified (Sturpe and Hermanns 1986; Molleman et al. 1993; Smirnov et al. 1992; Xiong et al. 1993), but TTX has no effect on the upstroke potential of the mouse small intestine and other tissues (Huizinga et al. 1991a; Barajas-López et al. 1989). Another sodium channel blocker, mexiletine, effectively blocked inward sodium current responsible for depolarization of the cardiac action potentials (Yotani and Akaike 1985; Hering et al. 1983). In the gastrointestinal tract, an in vivo investigation revealed that mexiletine reduced gastric, but not jejunal, slow wave frequency (Bisieledt and Bass 1991). In the mouse small intestine we observed that mexiletine did not alter the slow wave. It is, therefore, unlikely that a TTX- or mexiletine-sensitive Na⁺ channel is involved in the generation of the slow waves.

A nonspecific cation channel is a likely candidate for mediating Na⁺ influx as part of the slow wave upstroke. In various intestinal smooth muscle tissues, nonspecific ion channels have been identified (Inoue 1991; Pacaud and Bolton 1991; Sims 1992; Bolton et al. 1988; Smirnov et al. 1992). Muscarinic stimulation activates nonspecific cation conductances in canine gastric smooth muscle (Sims 1992), in guinea pig ileum (Inoue and Iaenbrg 1990; Inoue 1991), in canine pyloric circular smooth muscle (Vogalis and Sanders 1990), as well as in the dog colon circular muscle (Lee et al. 1993b; Molleman and Huizinga 1993). It is active at the resting membrane potential and inhibited by Ni²⁺.

The electrical recordings in this study were made from cells that form an electrical syncytium including interstitial cells of Cajal as well as smooth muscle cells. Recent evidence indicates that slow waves in the mouse small intestine are generated by the interstitial cells of Cajal (Huizinga et al. 1995; Ward et al. 1994). It is interesting to note that the relationships between ICC and smooth muscle are different in various organs in the gastrointestinal tract as summarized recently (Thuneberg 1989; Thuneberg et al. 1995; Christensen 1992; Fausone-Pellegrini 1992 (review)). A most apparent difference between the colon and the small intestine is that the network of ICC responsible for pacemaking activity is located in the myenteric plexus in the small intestine (Hara et al. 1986; Huizinga et al. 1995; Ward et al. 1994) and in the submuscular plexus in the colon (Liu and Huizinga 1993; Berezin et al. 1988; Liu et al. 1994; Sanders et al. 1991).
This together with the differences in the ionic basis of the slow waves suggests that a single mechanism of slow wave generation is unlikely to be found throughout the gastrointestinal tract.

Acknowledgements
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References


Chapter Eight

Regulation of the electrical slow wave by IP₃ induced calcium release in the murine small intestine. John Malysz and Jan D. Huizinga. Submitted to: Am. J. Physiol.

Method and contributions

John Malysz performed all electrophysiological experiments, did all analysis of electrophysiological data, wrote the manuscript, was involved in study design and did most of the literature research. Dr. Jan D. Huizinga supervised the project, was involved in study design and writing.
Abstract

Slow waves determine the (maximal) frequency and propagation characteristics of contractions in the intestine, yet little is known about the mechanisms of the slow wave regulation. In the current study, we examined the roles of intracellular Ca\(^{2+}\) and of the IP\(_3\) sensitive Ca\(^{2+}\) release mechanism in the regulation of the slow wave in the murine small intestinal musculature. A standard microelectrode technique was used to record slow waves in the presence of L-type Ca\(^{2+}\) channel blockade (1-5 \(\mu\)M nifedipine or verapamil). The data suggests that intracellular Ca\(^{2+}\) cycling, sensitive to IP\(_3\), determines the frequency and amplitude of the slow waves. The above conclusion is based on the following experiments. First, BAPTA/AM, a cytosolic Ca\(^{2+}\) chelator, markedly reduced or abolished the slow waves. Second, CPA, an inhibitor of SR Ca\(^{2+}\) ATPase, decreased the slow wave frequency, duration, and the resting membrane potential. Third, caffeine and PLC inhibitors (neomycin and NCDC) abolished or inhibited the slow wave generation or frequency. Fourth, in the presence of CPA, carbachol (transiently), noradrenaline, and phenylephrine increased the slow wave frequency. Without CPA carbachol was ineffective, but noradrenaline and phenylephrine still produced, although much weaker, increases in the frequency. In addition, phenylephrine increased the upstroke amplitude and decreased the plateau amplitude. Pre-treatment of the tissues with prazosin (5 \(\mu\)M), a selective \(\alpha_1\)-adrenoceptor antagonist, blocked the effects of both phenylephrine and noradrenaline, hence, supporting the involvement of the \(\alpha_1\)-adrenoceptors known to be coupled to PLC. Fifth, thimerosal (transiently) and 2-
aminoethoxydiphenyl borate, modulators of the IP$_3$ induced Ca$^{2+}$ release, increased the slow wave frequency. In summary, these data are consistent with a role of the IP$_3$ sensitive Ca$^{2+}$ release being involved in the control of the slow wave frequency and amplitude. Furthermore, activation of the release mechanism under certain conditions may cause depolarization since this effect was observed following prolonged perfusion with carbachol, thimerosal and 2-aminoethoxydiphenyl borate.

**Introduction**

The motor patterns in the intestine governing the peristaltic contractions are in part regulated via a myogenic control mechanism involving the pacemaker slow wave activity. The slow wave or a sinusoid-like oscillation in the membrane potential is thought to be initiated by interstitial cells of Cajal (ICC) (25) (49), and it restricts action potential generation usually to the plateau phase of the slow wave. During the plateau phase or the crest of the slow wave, the membrane potential is brought very close to the level required for activation of L-type Ca$^{2+}$ channels involved in action potential generation. Since Ca$^{2+}$ influx via L-type Ca$^{2+}$ channels initiates contractile activity of the smooth muscle cells, the maximum frequency of contractions is controlled by the slow waves. In addition, the slow waves determine propagation characteristics of contractions in the intestine (50) (58).

Although the cellular origins of the slow wave are now fairly well understood, the ionic and regulatory mechanisms responsible for the slow wave generation still remain
obscure. In the canine colon, the slow waves were found to be relatively insensitive to the changes in the membrane potential (22), insensitive to the L-type Ca\(^{2+}\) channel blockers (23), and highly sensitive to the removal of extracellular Ca\(^{2+}\) (2) and to cytosolic levels of cAMP (22), an intracellular second messenger. Furthermore, the canine colonic slow wave frequency could be reduced with either cyclopiazonic acid (CPA), a selective inhibitor of the SR Ca\(^{2+}\) ATPase, and BAPTA/AM, an intracellular Ca\(^{2+}\) chelator (34). Hence, it was suggested that an intracellular metabolic process, which is sensitive to intracellular Ca\(^{2+}\) and to cAMP, triggers the canine colonic slow waves.

Much less is known about the regulatory mechanisms for the small intestinal slow waves. The temperature sensitivity of the slow waves as observed in the small intestines of rabbits (17), cats (14), and mice (J. Malysz and J. D. Huizinga, unpublished observation) suggests involvement of a metabolic process in the slow wave regulation. In addition, extracellular Ca\(^{2+}\) and Na\(^{+}\) have been shown to be essential for the generation of the slow waves in the murine (36), rabbit (17), feline (13), and canine (9) small intestines. The insensitivity of the slow waves to the L-type Ca\(^{2+}\) channel blockade as reported in the murine (36) (60), porcine (5), feline (14) and canine (9) small intestines points to involvement of a Ca\(^{2+}\) conductance other than that mediated by the L-type Ca\(^{2+}\) channels. Whether intracellular Ca\(^{2+}\) also plays a critical role in the regulation of the slow waves in the small intestine still remains to be established. Data in this study provide such evidence in the murine small intestine.

Intracellularly, the major sites for Ca\(^{2+}\) storage are IP\(_3\) and ryanodine (or caffeine) sensitive Ca\(^{2+}\) stores residing in the sarcoplasmic reticulum (SR). Release of Ca\(^{2+}\) from
these stores is highly regulated and involves Ca\(^{2+}\) channels in the SR membrane. In the case of the IP\(_3\) sensitive Ca\(^{2+}\) stores, unique Ca\(^{2+}\) channels to which IP\(_3\) binds and causes their opening have been purified from the bovine aortic microsomes (10) and from membranes of the rat vas deferens smooth muscle (42) having molecular weights of 224,000 - 260,000 Da. In intestinal smooth muscle cells, existence of the IP\(_3\) sensitive Ca\(^{2+}\) stores has also been demonstrated. In permeabilized intestinal smooth muscle preparations, the addition of IP\(_3\) caused release of Ca\(^{2+}\) from the intracellular Ca\(^{2+}\) stores (28) (30), and specific high and low affinity IP\(_3\) binding sites were observed in microsomal fractions of the guinea pig ileal longitudinal smooth muscle cells (67). Functionally, the release of Ca\(^{2+}\) from the IP\(_3\) sensitive Ca\(^{2+}\) stores in smooth muscle cells can be involved in pharmacomechanical coupling leading to the generation of contractile responses during activation of muscarinic and histamine receptors (41) (55). An interesting aspect of the regulation of IP\(_3\) sensitive Ca\(^{2+}\) release involves intracellular Ca\(^{2+}\). Iino (27) showed that intracellular Ca\(^{2+}\) levels ranging from 0 to 300 nM enhance the release, while those above 300 nM inhibit the release in skinned smooth muscle cells of the guinea pig teania coli.

The present study set out to determine whether intracellular Ca\(^{2+}\) and intracellular Ca\(^{2+}\) stores, particularly of the IP\(_3\) type, are involved in the regulation of the slow wave in the murine small intestine. A major obstacle that this study had to overcome is the lack of selective pharmacological agents acting on the IP\(_3\) receptor. Two of the most commonly utilized pharmacological agents known to act on the IP\(_3\) induced Ca\(^{2+}\) release are IP\(_3\), an activator of the receptor, and heparin, a blocker of the receptor or channel. Due to their
plasma membrane impermeability, neither could be used in this study. Instead, this study relied on other agents known to act by different mechanisms on the IP$_3$ signalling pathway leading to the release of Ca$^{2+}$ from the intracellular Ca$^{2+}$ stores.

**Methods**

*Preparation and recording of the electrical activities*

A standard microelectrode technique, previously described in detail elsewhere (24) (37), was used to record slow waves from the murine small intestine. Adult mice used in this study were of either sex and supplied by Charles River Laboratories (CD1, St-Constant, Que.). The animals were cared for in accordance with the principles and guidelines of the Canadian Council on Animal Care.

Throughout the experiments, the L-type Ca$^{2+}$ blockers (1-5 μM nifedipine or verapamil) were present. As previously reported in detail in our previous studies on the murine small intestinal musculature (24) (37), a major effect of the blockade is to attenuate the generation of spike-like action potentials superimposed onto the plateau phases of the slow waves. In contrast, the slow waves are largely resistant to the L-type Ca$^{2+}$ channel blockade (24) (37). Consequently, mechanisms of the regulation and generation of slow waves can be studied as in this study in the presence of the L-type Ca$^{2+}$ channel blockade (verapamil or nifedipine).
Solutions and drugs

The composition of Krebs solution was (in mM): NaCl, 120.3; KCl, 5.9; CaCl₂, 2.5; MgCl₂, 1.2; NaHCO₃, 20.0; NaH₂PO₄, 1.2; and glucose, 11.5. The pH was 7.30 – 7.35. Other chemicals used were verapamil (Sigma Chemical Corp. St. Louis, Mo.), nifedipine (Sigma), BAPTA/AM (Molecular Probes Inc., Eugene, Oregon), CPA (cyclopiazonic acid, Sigma), neomycin (Sigma), NCDC (2-nitro-4-carboxyphenyl-N,N-diphenylcarbamate, Sigma), caffeine (Sigma), forskolin (Sigma), SNP (sodium nitroprusside, Sigma), noradrenaline (Sigma), phenylephrine (Sigma), prazosin (Sigma), carbachol (Sigma), thimerosal (Sigma), and 2APB (2-aminoethoxydiphenyl borate, Sigma).

Data analysis

Data were obtained usually from the same cells, before and in the presence of a drug. A difference was considered to be significant at P < 0.05. Data are expressed as means ± SEM, with n being the number of different animals used. Paired or unpaired Student’s t tests, whenever appropriate, were employed for comparison of the electrical activities determining the effects of drugs.
Figure 1. Summary of effects of pharmacological treatments on the slow wave in the murine small intestine. The abbreviations and concentrations are: 25 μM BAPTA/AM (n = 5), 1-3 μM cyclopiazonic acid or CPA (n=11), 4 mM neomycin (n = 7), 0.1 mM NCDC (n = 6), 0.3 mM NCDC (n = 7), 10 mM caffeine (n = 8), 1 μM forskolin (n = 7), 1 μM SNP (n = 7), 1 μM noradrenaline (NorA) (n = 11), 10 μM noradrenaline (n = 6), 3 μM phenylephrine (Phen) (n = 19), 10 μM phenylephrine (n = 12), 5 μM prazosin (n = 9), 0.5 μM carbachol (Carb) (n = 6), 1 μM carbachol (n = 5), 50 μM thimerosal (Thimer) (n = 5), 50 μM 2APB or 2-aminoethoxydiphenyl borate (Diphenbor) (n = 8), 9.2 mM extracellular K⁺ (n = 7), and 13.8 mM extracellular K⁺ (n = 7). The filled bars are controls and hatched bars experimental treatments. All experiments were carried out in the presence of L-type Ca²⁺ channel blockade (1-5 μM nifedipine or verapamil).

* P<0.05, ** P<0.01.
Results

Effect of chelation of intracellular Ca\(^{2+}\)

To determine if cytosolic Ca\(^{2+}\) was in any way involved in the generation of slow waves or in the regulation of the slow wave frequency, the effects of BAPTA/AM, a membrane permeable Ca\(^{2+}\) chelator, were studied. In this study, perfusion of tissues with the chelator at 25 μM led to either complete abolition of the slow wave in 2 out of 5 experiments or to marked reductions in the slow wave amplitude (by 58.1 ± 4.6 %), average rate of rise of the upstroke (by 70.7 ± 8.0 %), and the frequency (by 21.9 ± 10.6 %) in the remaining 3 experiments (see figures 1 and 2a). In one experiment in which the slow wave amplitude and the frequency were respectively decreased from 15.8 to 5.4 mV and from 26 to 15 cpm, the slow wave was abolished by a subsequent addition of 50 μM BAPTA/AM. In the other two experiments, no further increases were made in the BAPTA/AM concentration. Furthermore, it required a prolonged perfusion of at least 30 minutes for the effect of BAPTA/AM to be observed, and the effects of the chelator were not reversible.

Effect of inhibition of SR Ca\(^{2+}\) ATPase pump with cyclopiazonic acid (CPA)

Cyclopiazonic acid (CPA) is a selective inhibitor of the SR Ca\(^{2+}\) -ATPase (16) (20). An effect of CPA on the slow wave would indicate a role of the intracellular Ca\(^{2+}\) stores in the regulation of the slow wave. CPA (1-3 μM) transiently depolarized the cells by up to 20 mV, and it became technically difficult to maintain stable impalements
**Figure 2.** Effects of chelation of cytosolic Ca$^{2+}$, inhibition of SR Ca$^{2+}$ ATPase, inhibition of PLC activity and response to caffeine in the murine small intestine. (a) The top trace shows the control electrical activity and the lower trace the effect of 25 µM BAPTA/AM following 30 minutes of perfusion. Both traces were recorded from the same cell. (b) Top and lower traces in this panel respectively show the electrical activity recorded prior and 30 minutes after the addition of CPA (2 µM). (c) The traces on the left are controls with the middle trace being recorded also in the presence of CPA (3 µM). The traces on the right illustrate the effects of 4 mM neomycin (top and middle traces) and of 0.3 mM NCDC (bottom trace). Each of the experiments was obtained from a different mouse with the prior and after traces being from the same cells. (d) Top and lower traces were obtained from a continuous experiment from the same cell. At the arrow, 10 mM caffeine was added. There are no time lapses between the consecutive traces. Throughout the experiments in (a)-(d), L-type Ca$^{2+}$ channel blockers were present (1-5 µM nifedipine or verapamil).
during this phase. This was likely due to an increase in the cytosolic Ca\(^{2+}\) levels since studies on isolated intestinal myocytes showed that following the addition of CPA, the free cytosolic Ca\(^{2+}\) levels increased transiently then recovered close to the original resting Ca\(^{2+}\) levels (19)(43). Following the initial period of recording instability, it was possible to successfully impale cells. Effects of CPA as described below were obtained following at least 20 minute perfusion with this agent allowing for a sufficient recovery from the transient phase. As indicated in figures 1 and 2b, CPA (1-3 \(\mu\)M) significantly affected the slow wave amplitude, rate of rise of the upstroke, and the duration. In addition, marked reductions in the slow wave frequency were observed. In 11 experiments, CPA decreased the slow wave frequency by 46.1 ± 7.3 % (\(P < 0.001\)) from ~ 39.8 to 20.9 cpm. In 6 out of 11 experiments, the slow wave frequency reduced by more than 50 % (average decrease in these experiments: 63.8 ± 4.0 %); in other 3 experiments, the frequency did not decrease by more than 22 % even following prolonged perfusions of 30 minutes or more (average decrease in these experiments: 12.9 ± 4.9 %). In addition, the resting membrane potential was significantly depolarized by 6.2 ± 1.7 mV (\(n = 11\), \(P<0.006\)) ranging from a hyperpolarization of 4 mV to a depolarization of 19 mV (figure 1). It was important to establish whether the depolarization \textit{per se} with CPA might have caused the observed effects on the slow wave including that on the slow wave frequency. To test this possibility, we investigated the effects of increasing extracellular K\(^+\) on the slow wave, which produces depolarization. When extracellular K\(^+\) was increased from 4.6 to 9.2 mM, the membrane potential depolarized by 6.0 ± 1.5 mV (\(n = 7\)) (see figure 1). This level of depolarization was not significantly different from that observed with CPA (\(P = 0.95\)).
Similar reductions in the slow wave amplitude and the average rate of rise of the upstroke were observed with the two treatments. In contrast, reductions in the slow wave frequency and duration with CPA were significantly different from those identified with 9.2 mM $K^+$. CPA decreased the slow wave frequency by $46.1 \pm 7.3\%$ ($n = 11$), whereas the decrease with 9.2 mM $K^+$ was $15.1 \pm 3.6\%$ ($n = 7$, $P < 0.02$); the reduction in the slow wave duration by CPA was $26.0 \pm 5.1\%$ ($n = 11$) significantly different from that by 9.2 mM $K^+$, which was $6.2 \pm 0.4\%$ ($n = 7$, $P < 0.05$). Hence, the effects of CPA on the slow wave frequency and duration were at least in part independent from the effects on the membrane potential; however, depolarization per se with CPA likely produced the observed reductions in the slow wave amplitude and in the average rate of rise of the upstroke.

*Effect of phospholipase C inhibition with neomycin and 2-nitro-4-carboxylphenyl-$N,N$-diphenylcarbamate (NCDC)*

In order to further characterize the nature of the intracellular $Ca^{2+}$ stores, experiments were carried out with known inhibitors of the phosphatidylinositol specific PLC, neomycin and NCDC. Inhibition of the enzyme is expected to limit the generation of $IP_3$ (52) (59). As illustrated in figures 1, 2c and 3, neomycin (4 mM) and NCDC (0.1 and 0.3 mM) produced significant effects on the slow wave activity. Perfusion of the tissues with 4 mM neomycin ($n = 7$), which did not significantly affect the resting membrane potential or the slow wave duration, significantly modified the slow wave amplitude, average rate of rise of the upstroke and the frequency causing decreases of
Figure 3. Effects of pharmacological treatments on the slow wave in the presence of CPA in the murine small intestine. The abbreviations and concentrations are: 4 mM neomycin (n = 5), 2 μM noradrenaline (NorA) (n = 6), 3 μM phenylephrine (Phen) (n = 7), 5 μM prazosin (Praz) (n = 6), transient effects of 1 μM carbachol observed in less than 2 minutes of perfusion (Carb < 2 min) (n = 7), effects of 1 μM carbachol observed at perfusion of more than 2 minutes (Carb > 2 min) (n = 7). The filled bars are control and open bars experimental treatments. All experiments were carried out in the presence of CPA (1-6 μM CPA) and L-type Ca\(^{2+}\) channel blockade (1-5 μM nifedipine or verapamil).

* P<0.05, ** P<0.01.
21.2 ± 8.5 % (P < 0.05), 30.2 ± 11.9 % (P < 0.05), 27.2 ± 8.8 % (P < 0.05), respectively (figure 1 and 2c). If, however, neomycin (4 mM) was added in the presence of CPA where the slow wave frequency was already reduced, then this agent completely abolished the slow waves in 4 out of 5 experiments (figures 2c and 3). In the one experiment where the slow wave was not abolished, the slow wave frequency was reduced from 18.0 to 0.4 cpm and the amplitude from 10.7 to 3.4 mV.

NCDC at 0.3 mMabolished the slow wave activity in 4 out of 6 experiments. In the remaining two experiments, marked reductions in the slow wave amplitude (by 77 and 76 %), average rate of rise of upstroke (by 81 and 77 %), and the frequency (by 6 and 29 %) were observed. NCDC at 0.1 mM (n = 6) had only significant effects on the amplitude and the rate of rise producing reductions of 27.8 ± 7.9 % (P < 0.03) and 25.4 ± 9.8 % (P < 0.03), respectively. Effects of NCDC were not associated with significant effects on the resting membrane potential.

Effect of caffeine

Caffeine at high concentrations (10-20 mM) is known to block the IP₃ induced Ca²⁺ release (7)(45). In this study, we investigated the effects of 10 mM caffeine on the electrical slow wave. As exemplified in figure 2d and summarized with data in figure 1, the addition of caffeine (10 mM) led to a transient hyperpolarization lasting for 1-3 minutes followed by the recovery in the membrane potential close to the original level. At this time, the slow waves were either completely abolished or marked reductions in the slow wave frequency and amplitude were observed. Prolonged perfusion with
caffeine of 10 minutes or more led to a marked depolarization of \(14.0 \pm 3.4\) mV (\(n = 11, P < 0.01\)) and complete abolition of the slow wave. As in the case with CPA, we were concerned that the depolarization \textit{per se} with caffeine could have abolished the slow waves. In order to test this possibility, the extracellular K\(^+\) was increased until comparable depolarizations were observed. It was determined that increasing the extracellular K\(^+\) from 4.6 to 13.8 mM depolarized the tissues by 13.7 \pm 2.3 mV (\(n = 7\)). This depolarization was not significantly different than that observed with 10 mM caffeine (\(P = 0.94\)). However, in contrast to the effect of caffeine, increasing the extracellular K\(^+\) by 9.2 mM did not abolish the slow waves. In seven experiments, the slow waves remained with the slow wave characteristics in the presence of 13.8 mM extracellular K\(^+\) summarized in figure 1. Consequently, the abolition of the slow waves with caffeine was likely independent from the depolarization.

\textit{Effects of forskolin and SNP}

One of the known effects of caffeine is to inhibit cytosolic nucleotide phosphodiesterases (11) (54). This leads to increases in cytosolic levels of cAMP and cGMP and to the subsequent activation of PKA and PKG signalling pathways. In order to investigate whether the observed effects of caffeine on the slow wave could have been due to increases in intracellular levels of cAMP or cGMP, the effects of forskolin and SNP, which respectively increase intracellular levels of cAMP and cGMP, were tested on the slow wave (53) (68). As illustrated in figure 1, neither could mimic the effects of caffeine on the slow wave except for the hyperpolarization. 1 \(\mu\)M forskolin and 1 \(\mu\)M
SNP hyperpolarized the tissues by $13.8 \pm 2.9$ mV ($n = 7$, $P < 0.004$) and $6.3 \pm 1.2$ mV ($n = 7$, $P < 0.003$), respectively. SNP also significantly increased the slow wave amplitude ($11.0 \pm 3.4$ %, $n = 7$, $P < 0.03$) and the average rate of rise of the upstroke ($24.9 \pm 0.3$ %, $n = 7$, $P < 0.003$). Forskolin (1 μM) affected the slow wave amplitude (decrease of $4.8 \pm 1.9$ %, $n = 7$, $P < 0.04$) and the frequency (increase of $10.2 \pm 2.2$ %, $n = 7$, $P < 0.002$).

**Effect of α₁-adrenoceptor activation**

The above experiments suggest a potential role of IP₃ in the regulation or in the generation of the slow wave. The role of this second messenger was further examined by determining the effects of agents known to stimulate IP₃ generation in intestinal smooth muscle cells. In this study, we utilized noradrenaline and phenylephrine. Both can activate α₁-adrenoceptors, which are known to be coupled to PLC via G-protein dependent mechanisms (18) (40). As summarized in figure 3 and exemplified in figure 4, in the presence of CPA (1-3 μM) where the slow wave frequency was reduced by at least 40 %, the application of noradrenaline (2 μM) and phenylephrine (3 μM) increased the slow wave frequency by $69.5 \pm 28.3$ % ($n = 6$, $P < 0.002$) and $78.8 \pm 32.4$ % ($n = 7$, $P < 0.04$), respectively. No other slow wave characteristics were affected (figure 3). These increases in the slow wave frequency were prevented by pretreatment of the tissues with prazosin, a selective α₁-adrenoceptor antagonist. Prazosin (5 μM), while having no effect on the slow wave in the presence of CPA (figure 3), completely inhibited the responses to noradrenaline (2 μM) and phenylephrine (3 μM) (table I).
Figure 4. Effects of adrenergic agents on the slow wave in the murine small intestine. The top traces in (a)-(d) are controls with the top trace in (b) being recorded in the presence of prazosin (5 μM), and top traces in (c) and (d) recorded in the presence of CPA, 2 μM in (c) and 1 μM in (d). Lower traces illustrate the effects of 10 μM phenylephrine (a), 10 μM phenylephrine in the presence of prazosin (5 μM) (b), 2 μM noradrenaline in the presence of CPA (2 μM) (c), 3 μM phenylephrine in the presence of CPA (1 μM) (d). Each of the experiments was obtained from a different mouse with the prior and after traces being obtained from the same cells. There are 10-15 minutes lapses in (a) and (b) and 3-5 minute lapses in (c) and (d). Throughout the experiments in (a)-(d), L-type Ca^{2+} channel blockers were present (1-5 μM nifedipine or verapamil).
Table I. Effect of phenylephrine and noradrenaline on the slow wave in the presence of prazosin in the murine small intestine.

<table>
<thead>
<tr>
<th></th>
<th>Resting membrane potential (mV)</th>
<th>Average Amplitude (mV)</th>
<th>Average rate of rise of upstroke (mV/s)</th>
<th>Average Frequency (cpm)</th>
<th>Average Duration (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 μM prazosin (n=7)</td>
<td>-61.1±3.6</td>
<td>19.6±2.1</td>
<td>121.4±23.2</td>
<td>38.0±1.9</td>
<td>0.59±0.05</td>
</tr>
<tr>
<td>10 μM phenylephrine</td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>prazosin (5μ M) (n=7)</td>
<td>-61.6±4.0</td>
<td>20.0±2.4</td>
<td>127.1±22.8</td>
<td>37.1±1.8</td>
<td>0.62±0.06</td>
</tr>
<tr>
<td>5 μM prazosin in CPA (n=4)</td>
<td>-55.5±2.6</td>
<td>6.3±1.1</td>
<td>56.2±17.7</td>
<td>16.3±3.2</td>
<td>0.45±0.07</td>
</tr>
<tr>
<td>2 μM noradrenaline in</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 μM prazosin in CPA (n=4)</td>
<td>-52.8±2.1</td>
<td>7.7±1.0</td>
<td>57.9±13.9</td>
<td>17.8±3.9</td>
<td>0.42±0.05</td>
</tr>
<tr>
<td>5 μM prazosin in CPA (n=4)</td>
<td>-53.0±3.5</td>
<td>12.1±5.2</td>
<td>94.1±21.3</td>
<td>15.9±2.8</td>
<td>0.35±0.04</td>
</tr>
<tr>
<td>3 μM phenylephrine in</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 μM prazosin in CPA (n=4)</td>
<td>-51.3±2.8</td>
<td>11.6±6.3</td>
<td>91.4±26.0</td>
<td>20.0±5.1</td>
<td>0.34±0.03</td>
</tr>
</tbody>
</table>

CPA concentrations: 1-3 μM
In other experiments, the effects of noradrenaline (1 and 10 μM) and phenylephrine (1 and 10 μM) were determined without CPA. As shown in figure 1, the addition of both caused similar, but not identical, effects on the slow wave activity. Increases in the slow wave frequency were usually observed although to a small extent by 5.7 ± 1.8 % (n = 19, P < 0.005), 7.0 ± 1.8 % (n = 12, P < 0.005), 4.1 ± 1.6 % (n = 11, P < 0.04), and 5.1 ± 1.7 % (n = 6, P < 0.05) for 3 and 10 μM phenylephrine, and 1 and 10 μM noradrenaline, respectively. However, the clear increase in the slow wave frequency was not detected in all experiments. For example, in 4 out of 11 experiments with 1 μM noradrenaline, and in 8 out of 19 experiments with 3 μM phenylephrine, no increases in the slow wave frequency could be observed. Additionally, phenylephrine and noradrenaline affected the slow wave amplitude. In order to describe the effects of the adrenoceptor agonists on the amplitude, it became necessary to make a distinction between the upstroke amplitude and plateau amplitude. In other experiments described in this manuscript, the plateau amplitude was affected to the same degree as the upstroke amplitude, and only the upstroke amplitude (i.e. the slow wave amplitude in figure 1) was reported. As exemplified in figure 4a, 3 and 10 μM phenylephrine decreased the plateau amplitude but increased the upstroke amplitude. The plateau amplitude was decreased from 17.0 ± 1.3 to 14.6 ± 1.5 mV (n = 19, P < 0.02) with 3 μM phenylephrine and from 17.3 ± 0.9 to 11.8 ± 0.9 mV (n = 12, P < 0.0001) with 10 μM phenylephrine. In comparison, the upstroke amplitude (slow wave amplitude in figure 1) increased from 19.3 ± 1.0 to 20.5 ± 1.0 mV (n = 19, P < 0.05) with 3 μM phenylephrine and from 20.3 ± 1.3 to 22.1 ± 1.3 mV (n = 12, P < 0.01) with 10 μM phenylephrine. In comparison to the
effects of phenylephrine, noradrenaline did not increase the upstroke amplitude (slow wave amplitude in figure 1), but it also decreased the plateau amplitude. At 1 μM noradrenaline, the plateau amplitude decreased from $17.4 \pm 2.2$ to $14.6 \pm 2.4$ mV ($n = 11$, $P < 0.05$) and at 10 μM noradrenaline from $16.6 \pm 0.7$ to $11.1 \pm 0.8$ mV ($n = 6$, $P < 0.01$). A concentration of 1 μM noradrenaline had no effect on the upstroke amplitude (slow wave amplitude in figure 1), whereas the effect of 10 μM noradrenaline just failed to reach the statistical significance ($n = 6$, $P = 0.053$). Closer examination of the data indicates that in all but one experiment the upstroke amplitude decreased. In the remaining experiment, it increased from $20.9$ to $22.3$ mV. If data from the single experiment are removed from the analysis, then the averages for the upstroke amplitude are $21.1 \pm 1.5$ mV (for control) and $15.2 \pm 1.7$ mV (for 10 μM noradrenaline) reaching the significance ($n = 5$, $P < 0.03$). Hence, the slow wave upstroke amplitude was increased by phenylephrine, but not by noradrenaline. In addition, as indicated in figure 1, phenylephrine decreased the slow wave duration and increased the average rate of rise of the upstroke, and both phenylephrine (3 and 10 μM) and noradrenaline (10 μM) produced significant hyperpolarizations. The respective hyperpolarizations were $2.5 \pm 0.7$ mV ($n = 19$, $P < 0.002$), $3.4 \pm 0.8$ mV ($n = 12$, $P < 0.003$), and $8.8 \pm 1.0$ mV ($n = 6$, $P < 0.0006$). It was unlikely that any of the observed effects, especially for phenylephrine, were due to the hyperpolarization since cromakalim, a $K_{\text{ATP}}$ channel opener, at 1 μM produced a hyperpolarization of $8.6 \pm 1.4$ mV ($n = 11$, $P < 0.0001$), but it did not significantly affect any slow wave characteristics (data not shown).
In order to confirm that the observed effects of phenylephrine were due to the activation of the $\alpha_1$-adrenoceptors, experiments were carried out with phenylephrine on tissues previously pretreated with prazosin (5 $\mu$M), a selective $\alpha_1$-adrenoceptor antagonist. As indicated in figure 1, prazosin on its own had no effects on the slow wave when perfused for up to 15 minutes. And as summarized in table 1 and exemplified in figure 4b, phenylephrine (10 $\mu$M) in the presence of prazosin (5 $\mu$M) did not produce any effects. Hence, this indicates that the effects of phenylephrine on the slow wave were most likely due to the activation of the $\alpha_1$-adrenoceptors mediating increases in the slow wave frequency as well as affecting the upstroke and plateau amplitudes.

*Effect of carbachol*

Carbachol induces IP$_3$ synthesis via activation of the M$_3$ receptors in the intestinal smooth muscle (8) (51). As illustrated in figures 3 and 5a, the addition of carbachol (1 $\mu$M) in the presence of CPA resulted in a significant increase of the slow wave frequency by 99.3 $\pm$ 35.4 % ($n = 7$, $P < 0.05$). This increase was detected during up to 2 minutes of the perfusion with carbachol. In addition, during this transient phase in 7 experiments, significant effects on the resting membrane potential (depolarization of 2.5 $\pm$ 0.7 mV, $P < 0.02$), the amplitude (decrease of 13.8 $\pm$ 4.3 %, $P < 0.02$) and the average rate of rise (decrease of 20.6 $\pm$ 5.8 %, $P < 0.02$) were observed (figure 3). Although the effects on the amplitude and the average rate of rise could be explained by the effect of depolarization *per se*, the effect on the slow wave frequency was clearly not dependent on the depolarization. As explained above, pharmacological depolarization never increased the
Figure 5. Effects on the slow wave with agents known to act on the IP₃ signaling pathway in the murine small intestine. (a) This single trace was recorded in the presence of 1 μM CPA. At the arrow, 1 μM carbachol was added. (b) The three traces are from the same experiment obtained from a single cell. The first, top trace was obtained prior to the addition of 50 μM thimerosal, the second (middle) trace 2 minutes after, and the third trace 5 minutes after the addition of thimerosal. (c) The first, top trace illustrates the control electrical activity prior to the addition of 50 μM 2-aminoethoxydiphenyl borate. The second and third traces were obtained following 10 and 12 minute perfusion with the agent, respectively. Traces are from a continuous experiment. Throughout the experiments in (a)-(c), L-type Ca²⁺ channel blockers were present (1-5 μM nifedipine or verapamil).
slow wave frequency. In the prolonged presence of carbachol, the resting membrane potential continued to depolarize. Associated with the depolarization were reductions in the slow wave amplitude, average rate of rise of the upstroke, and the frequency. All these effects may be explained by the effect of depolarization per se. Hence, the addition of carbachol in the presence of CPA transiently increased the slow wave frequency.

Additional experiments were carried out with carbachol (0.5 and 1 μM) without CPA. These concentrations of carbachol produced depolarizations (figure 1). However, in contrast to the effects of carbachol in the presence of CPA, no transient increases in the slow wave frequency were observed. In addition, associated with the depolarizations were significant effects on the amplitude, average rate of rise of the upstroke, frequency and the duration (see figure 1). As explained above, these effects on the slow wave were likely due to the depolarization.

_Effect of IP₃ receptor sensitization or activation with thimerosal and 2-aminoethoxydiphenyl borate_

Thimerosal has been shown to cause sensitization of the IP₃ induced Ca²⁺ release (4) (29). In this study, we tested an effect of 50 μM thimerosal on the slow wave activity. This agent produced effects in two phases as exemplified in figure 5b. In the first phase lasting 1-3 minutes following the onset of perfusion, it caused a depolarization of 7.0 ± 1.2 mV (n = 5, P < 0.03). Other effects on the slow wave during this phase are summarized in figure 1, and they depict an increase in the slow wave frequency (by 16.4 ± 4.2 %, P < 0.03) as well as decreases in the slow wave amplitude (by 24.6 ± 6.5 %, P <
0.05), average rate of rise of the upstroke (by 34.8 ± 9.5 %, P < 0.05), and the duration (by 22.1 ± 3.8 %, P < 0.04) in 5 experiments. All but the effect on the frequency can be explained by the effect of depolarization since the depolarization per se does not increase the slow wave frequency (see above). In the second phase, the membrane potential continued to depolarize by up to ~ 30 mV. At this time, the slow waves were either no longer generated or had very small amplitudes (see figure 4b). This effect on the slow wave may be explained by the effect of thimerosal on the membrane potential as a similar extent of depolarization (~ 20 mV) was produced when the extracellular K⁺ was increased by 4-5 times normal levels (i.e. abolition of the slow wave or very small amplitude slow waves, data not shown).

Recently, 2-aminoethoxydiphenyl borate (2APB) was suggested to act on the IP₃ induced release (39). In our study, an effect of 50 µM 2APB was tested. In 8 experiments, 2APB increased the slow wave frequency by 7.4 ± 2.2 % (P < 0.02) (figures 1 and 5c). Other slow wave characteristics were also affected including the resting membrane potential (depolarization of 7.4 ± 2.2 mV, P < 0.009), amplitude (decrease of 40.4 ± 4.9 %, P < 0.002), and the average rate of rise of the upstroke (decrease of 38.2 ± 8.6 %, P < 0.008). However, in contrast to the effect on the frequency, these effects could be explained by the effect of depolarization per se.

Discussion

This study provides evidence for cytosolic Ca²⁺ and intracellular Ca²⁺ stores in regulating the generation of electrical slow waves in the murine small intestine. Perfusion
of tissues with BAPTA/AM, an intracellular Ca\(^{2+}\) chelator, and CPA, an inhibitor of the SR Ca\(^{2+}\) ATPase pump, reduced the slow wave frequency or amplitude. Furthermore, studies with agents known to act on the IP\(_3\) signalling pathway including caffeine, neomycin, NCDC, noradrenaline, phenylephrine, carbachol and 2APB suggest the involvement of the IP\(_3\) sensitive Ca\(^{2+}\) stores in the regulation of the slow wave. Both neomycin and NCDC function as inhibitors of phosphatidylinositol specific PLC, and they were reported to inhibit agonist stimulated IP\(_3\) generation in ileal longitudinal smooth muscle cells (65), in atrial cells (21), in pancreatic acinar cells (56), and in urinary smooth muscle (4). In this study, both PLC inhibitors, neomycin and NCDC, attenuated the slow wave generation (i.e. produced reductions in the slow wave amplitude and frequency). Effects of NCDC were more pronounced leading to the abolition of the slow wave; neomycin had a similar effect only in tissues pretreated with CPA in which the slow wave frequency was markedly reduced. Similar effects on the slow wave with neomycin were previously reported for the canine colon (34).

Two receptors that have been linked to the activation of PLC and the generation of IP\(_3\) in various systems are muscarinic receptors (M\(_3\)) and adrenoceptors (\(\alpha_1\)). Muscarinic receptor activation with either carbachol or acetylcholine in ileal, gastric, and colonic myocytes (30) (31) (35) (63) as well as stimulation of the \(\alpha_1\)-adrenoceptors by either phenylephrine, a selective \(\alpha_1\)-adrenoceptor agonist, or noradrenaline, an \(\alpha\)- and \(\beta\)-adrenoceptor agonist (32) (35), in vascular smooth muscle cells caused release of Ca\(^{2+}\) from the IP\(_3\) sensitive Ca\(^{2+}\) stores and subsequent contractile responses. Similarly in smooth muscle of the stomach (12) and small intestine (6), the selective activation of \(\alpha_1\)-
adrenoceptors led to increases in contractility. In this study, the activation of the muscarinic receptors and α1-adrenoceptors in the presence of CPA increased the slow wave frequency by ~70-100 %. Phenylephrine without CPA, although rather weakly, also increased the slow wave frequency as well as the upstroke amplitude, and it decreased the plateau amplitude. The last effect may be explained by the activation of the outward K⁺ current by phenylephrine as previously reported for intestinal myocytes (3) or by the effect of the high frequency. In the canine antral musculature, artificial shortening of the interval between slow waves essentially produced the same effect as observed with phenylephrine in the current study i.e. reduction of the plateau amplitude (48). In comparison, carbachol without CPA did not (transiently) increase the slow wave frequency, instead a reduction in the frequency as well as decreases in the amplitude and rate of rise of the upstroke were observed. Although these effects may be explained by the effect of depolarization per se, other possible mechanisms contributing to the observed effects of carbachol might be: inhibition of the IP₃ receptor by the elevated intracellular Ca²⁺ and activation of the PKC signalling pathway. The former explanation is based on a study by Iino (27) illustrating that intracellular Ca²⁺ levels of 0-300 nM enhanced the IP₃ mediated release, while concentrations higher than 300 nM inhibited the release. Hence, carbachol might have released enough intracellular Ca²⁺ to inhibit the IP₃ receptor. The second explanation refers to the other second messenger pathway stimulated when PLC is activated as for example with acetylcholine or carbachol, the DAG mediated activation of PKC. In the canine antral musculature, simulation of the PKC signalling pathway decreased the slow wave frequency, amplitude and the duration
without affecting the resting membrane potential (44). Interestingly, the activation of muscarinic receptors with acetylcholine or carbachol or stimulation of gastrin receptors with pentagastrin increased the slow wave frequency in the antral musculature (57) suggesting that the PKC pathway does not completely prevent the increases in the slow wave frequency.

Our explanation for the strong increases in the slow wave frequency in the presence of CPA as observed with noradrenaline, phenylephrine, or carbachol, in comparison to weak or no increases in the frequency with these agents without CPA, is that under normal physiological and recording conditions (i.e. without CPA), the slow wave frequency was already near its maximal achievable frequency and could not be readily increased by these agents. In contrast in the presence of CPA, when the slow wave frequency was markedly reduced and, hence, artificially moved away from its physiological maximum, marked increases in the slow wave frequency were possible. In comparison, the antral musculature appears to have its physiological slow wave frequency much lower than the achievable maximum since carbachol, acetylcholine, and pentagastrin (57) readily increased the slow wave frequency without the need for CPA.

Other agents acting on the IP$_3$ signalling pathway are thimerosal, 2APB, and caffeine. Thimerosal is thought to sensitize the IP$_3$ induced Ca$^{2+}$ release mechanism (4) (29). In pancreatic acinar cells, thimerosal induced Ca$^{2+}$ release from intracellular Ca$^{2+}$ stores that was blocked by heparin, a blocker of the IP$_3$ receptor (61). In smooth muscle cells, thimerosal is also believed to have the same effect on the IP$_3$ receptors although other mechanisms by which thimerosal activates intracellular Ca$^{2+}$ release have been
proposed as well (38) (46). Recently, 2APB was described by Maruyama et al. (39) as a novel membrane permeable modulator of the IP₃ induced Ca²⁺ release. They showed that at concentrations greater than 90 μM, stimulation of the release occurred in the rat cerebral microsomal preparations. In the current study, perfusion of tissues with 2APB and thimerosal (for up to 3-5 minutes) increased the slow wave frequency, decreased the slow wave amplitude and the rate of rise of the upstroke, and produced depolarizations. All but the effect on the frequency could be explained by the effect of depolarization. Furthermore, the fact that stimulation or sensitization of the IP₃ induced Ca²⁺ release produced depolarization suggests that Ca²⁺ release via the IP₃ receptors is also involved in the regulation of the membrane potential.

Caffeine at high concentrations is known to block the IP₃ induced Ca²⁺ release (7) (15). In this study, 10 mM caffeine inhibited the slow wave apparently independently of the effect of depolarization although the change in the membrane potential was also prominent especially following more than 3 minutes of perfusion. In addition, caffeine (10 mM) produced a transient hyperpolarization during the initial 3 minutes of perfusion. It is likely that the hyperpolarizing and depolarizing effects of caffeine were due to effects other than that on the IP₃ induced Ca²⁺ release. The former effect was probably mediated by inhibition of intracellular cyclic nucleotide phosphodiesterases since in the murine small intestine SNP, which increases intracellular cGMP (68), and forskolin, which increases intracellular cAMP (53), as observed in this study and IBMX (J. Malysz and J. D. Huizinga, unpublished observation), which increases both cAMP and cGMP (66), produced hyperpolarizations as well. The depolarization could be explained by the
activation of the ryanodine receptors that results in the release of Ca\(^{2+}\) from the intracellular stores. Effects of high concentrations of caffeine (5-10 mM) were also previously studied on the antral (62) and colonic slow waves (34). In these tissues, caffeine inhibited the slow waves as well. However, the effect of caffeine on the antral and colonic slow waves may not only be explained by the inhibition of the IP\(_3\) induced Ca\(^{2+}\) release but also by the increases in cytosolic cAMP levels since in the two organs activation of the PKA signalling pathway led to the abolition of the slow waves or marked reductions in the slow wave amplitude or frequency (22) (62). In this respect, the murine small intestine offers an advantage in interpretation of the effects of caffeine. As judged in this study by the effects of forskolin and SNP, which respectively activate the PKA and PKG signalling pathways, the stimulation of the two pathways did not mimic the responses of caffeine on the slow wave except for the hyperpolarization.

As discussed above, agents acting on the IP\(_3\) signalling pathway culminating in the release of Ca\(^{2+}\) from the intracellular Ca\(^{2+}\) stores had complex effects on the slow waves including on the frequency, waveform, and the resting membrane potential. Out of the described effects only that on the slow wave frequency can be attributed with any certainty to a selective action on the pacemaker ICC. Since smooth muscle cells in tissue likely possess an active mechanism for slow wave regeneration (33), although lacking the triggering mechanism present in the ICC, smooth muscle cells can alter the slow wave waveform and the resting membrane potential. This is particularly relevant in our study since recordings were most likely made from smooth muscle cells rather than from the ICC.
The question as to how the IP₃ signalling controls the slow wave remains to be solved by future studies including those using isolated ICC. Based on the canine colonic studies, Liu et al. (34) proposed that the periodic release of Ca²⁺ from the intracellular stores in subplasmalemmal spaces either directly or indirectly opens a plasmalemmal pacemaker channel allowing for influx of Ca²⁺ via a non-L-type Ca²⁺ channel that subsequently causes the slow wave generation. The release of Ca²⁺ was proposed to be regulated by an IP₃ sensitive mechanism since neomycin and caffeine markedly reduced the slow wave frequency or amplitude independently of the membrane potential. However, neither neomycin nor caffeine are selective (47) (64). Our study on the murine small intestinal muscle also supports a role of the IP₃ induced Ca²⁺ release in the regulation of the slow wave. Furthermore, we provide even stronger evidence that relies on other agents in addition to neomycin and caffeine. The observation that mechanisms regulating the slow wave may be similar in the colon and the small intestine is also supported by studies showing that in both tissues ICC are thought to be responsible for the initiation of the slow waves (25) (49).

Our data also suggest that intracellular Ca²⁺ released via IP₃ receptors is involved in the regulation of the slow wave frequency and amplitude apparently via independent mechanisms for the following reasons. First, NCDC, a PLC inhibitor, had much stronger effects on the slow wave amplitude than on the frequency. Second, although the effects of CPA were highly variable, in some cases (e.g. figure 2b) CPA markedly reduced the slow wave frequency (by more than 50 %) with little effect on the amplitude. Third, in the presence of CPA, the activation of the α₁-adrenoceptors produced marked increases in
the frequency but no effects on the amplitude. Fourth, treatment of the tissues with BAPTA/AM, an intracellular Ca\(^{2+}\) chelator, when not abolishing the slow wave, markedly reduced the slow wave amplitude (~ 60 %) and only weakly the frequency (~ 22 %). A possible explanation for the differential sensitivities of the frequency and of the amplitude may be related to the selective effects of pharmacological agents on ICC or on smooth muscle cells. According to this proposal, changes in the slow wave frequency could be attributed to pharmacological treatments affecting the ICC, whereas those affecting the amplitude by acting on the smooth muscle cells. Alternatively, there may be two types of IP\(_3\) sensitive release mechanisms in ICC, one controlling the frequency and the other the amplitude. Clearly, studies on isolated ICC and smooth muscle cells will be needed to clarify these issues.

In summary, we provide evidence for a role of intracellular Ca\(^{2+}\) stores, IP\(_3\) sensitive Ca\(^{2+}\) release, and cytosolic Ca\(^{2+}\) in the regulation of the slow wave frequency and amplitude in the murine small intestine. The evidence is based on the effects of pharmacological agents with known actions on the IP\(_3\) signalling pathway leading to the release of Ca\(^{2+}\) from the intracellular stores.

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Chapter Nine


Method and contributions

John Malysz performed all electrophysiological experiments, did all analysis of electrophysiological data, wrote the manuscript and was involved in study design. He also did most of the literature research. Dr. Jan D. Huizinga supervised the project, was involved in study design and writing.
Abstract

Understanding the intracellular regulation of the intestinal pacemaker activity is critical before pharmacological strategies can be designed to alter the abnormal pacemaker activity. In the present study, the roles of the Ca\textsuperscript{2+} induced Ca\textsuperscript{2+} release (CICR) and Cl\textsuperscript{-} channels in the regulation of the electrical activity in the murine small intestine were investigated. Experiments were carried out in the presence of L-type Ca\textsuperscript{2+} channel blockade. Perfusion of the tissues with either ryanodine (50 \textmu M) or caffeine (5 mM), both expected to stimulate CICR, resulted in depolarizations of \sim 9 mV. Although other slow wave parameters were also affected, these effects can be explained by the effect depolarization \textit{per se} since similar results were observed when tissues were depolarized with high extracellular K\textsuperscript{+}. Investigation of possible mechanisms mediating the CICR induced depolarization revealed that Cl\textsuperscript{-} channels were not predominately involved for the following reasons. First, caffeine (5 mM) in the presence of low extracellular Cl\textsuperscript{-} solution (isethionate) did not produce a significantly greater depolarization as would be expected under this condition. Second, in the presence of niflumic acid (100 \textmu M) and DIDS (500 \textmu M), the addition of caffeine (5 mM) produced comparable depolarizations to those observed with caffeine alone. Of the K\textsuperscript{+} channels examined, K\textsubscript{DR} channels may be involved in the CICR induced depolarization since 4-AP (2 mM), a blocker of K\textsubscript{DR} channels, produced significant depolarizations, while blockers of K\textsubscript{Ca} channels (2 mM TEA and 40 nM penitrem A) were without any effects. Additional experiments with dantrolene (30 \textmu M), a blocker of CICR, indicated that this agent had significant effects on the slow wave frequency, amplitude, and rate of rise of
the upstroke but not on the resting membrane potential. In conclusion, our data suggest the following: pharmacological activation of CICR causes depolarization mediated by 4-AP sensitive $K^+$ channels without the involvement of $Cl^-$ channels, and the CICR is not involved in the initiation of the slow waves but may regulate the slow wave amplitude and frequency. Furthermore, the data showed that $Cl^-$ channels are not involved in the slow wave initiation since niflumic acid (100 $\mu$M) and DIDS (500 $\mu$M) did not abolish the slow wave generation although hyperpolarizations and significant reductions in the slow wave amplitude and rate of rise of the upstroke were observed. The latter findings suggest that $Cl^-$ channels may be involved in the generation of the slow wave amplitude or plateau.

Introduction

In the intestinal musculature, patterns of smooth muscle contractility are regulated in part by a myogenic control mechanism involving the intrinsic electrical slow wave activity (Diamant et al. 1969; Szurszewski, 1987). Under the slow wave control, the smooth muscle cells in situ oscillate between periods of high and low excitability. The former phase corresponds to the crest of slow wave where the membrane potential favors L-type $Ca^{2+}$ channel activation and $Ca^{2+}$ influx, while the later phase coincides with the trough of slow wave that creates an unfavorable condition for the channel activation. Hence, slow waves by regulating L-type $Ca^{2+}$ channel opening set the (maximum) frequency of contractions and, furthermore, control the propagation characteristics of contractions in the intestine (Diamant et al. 1969; Szurszewski, 1987).
Little is known about the regulatory mechanisms of the slow wave generation. In the small intestines of dogs (Cayabyab et al. 1996), cats (Dahms et al. 1987), rabbits (El-Sharkawy et al. 1975), and mice (Malysz et al. 1995), the slow wave frequency and amplitude are sensitive to, and apparently regulated by, extracellular Ca\(^{2+}\). Furthermore, differential sensitivities of the amplitude and frequency to Ca\(^{2+}\) suggest involvement of two separate Ca\(^{2+}\) regulated processes, none depending on the influx of Ca\(^{2+}\) via L-type Ca\(^{2+}\) channels. In the small intestines of rabbits (El-Sharkawy et al. 1975), dogs (Cayabyab et al. 1996), and mice (Malysz et al. 1995; Malysz et al. 1996), L-type Ca\(^{2+}\) channel blockers were without any effects on the slow wave, but they abolished the action potential generation on the plateau phase of the slow wave. Hence, the mechanisms of the slow wave initiation and regulation can be studied as in this study in the presence of L-type Ca\(^{2+}\) blockade.

Recent studies on the canine colon (Liu et al. 1995) and on the murine small intestine (Malysz et al. 1997) provided evidence consistent with the regulation of the slow wave by intracellular Ca\(^{2+}\) and intracellular Ca\(^{2+}\) stores associated with the IP\(_3\) induced Ca\(^{2+}\) release mechanism. In the present study, the importance of the Ca\(^{2+}\) induced Ca\(^{2+}\) release (CICR) was addressed. CICR involves exclusively ryanodine gated channels or receptors present in intracellular Ca\(^{2+}\) stores (SR or ER). Three types of ryanodine receptors (RyR1, RyR2, and RyR3) are currently recognized, each encoded by a separate gene named *ryr-1*, *ryr-2*, and *ryr-3* (McPherson et al. 1993; Coronado et al. 1994; Sorrentino et al. 1993). Due to predominant expression of one subtype in a given tissue, the type 1 ryanodine receptor (RyR1) is also referred to as the skeletal ryanodine
receptor, the type 2 ryanodine receptor (RyR2) as the cardiac ryanodine receptor, and the type 3 ryanodine receptor (RyR3) as the brain ryanodine receptor. In addition, type 1 ryanodine receptors are found in cerebellum and sea urchin eggs; type 2 ryanodine receptors in stomach, endothelial cells, and brain; and type 3 ryanodine receptors in epithelial cells, skeletal muscle, kidney, lung, stomach, spleen, ileum, jejunum, and brain (McPherson et al. 1993; Coronado et al. 1994; Sorrentino et al. 1993).

The role of CICR in gut smooth muscle cells remains largely unknown. However, functional studies have demonstrated the presence of ryanodine sensitive Ca\(^{2+}\) stores in these smooth muscle cells. The evidence is based on studies measuring \(^{45}\)Ca\(^{2+}\) release from intracellular pools (Kuemmerle et al. 1994; Obara et al. 1987), binding of \(^{3}H\)ryanodine (Kuemmerle et al. 1994; Wibo et al. 1994), intracellular Ca\(^{2+}\) measurements utilizing Ca\(^{2+}\) imaging methods (Oh et al. 1997; Iino et al. 1988; Iino, 1989), and smooth muscle contractility (Oh et al. 1997; Obara et al. 1987). In addition, the ryanodine receptor has been purified from the stomach smooth muscle of the toad \textit{Bufo marinus} (Xu et al. 1994), and a RT-PCR method demonstrated the presence of mRNA for the type 3 ryanodine receptor in muscularis propria of the murine small intestine (Bielefeldt et al. 1997).

Ca\(^{2+}\) release via ryanodine gated channels localized to specific areas of the cytosol close to the plasma membrane has been shown to activate \(K_{Ca}\) and \(Cl_{Ca}\) channels in intestinal smooth muscle cells (Bolton et al. 1996; Komori et al. 1991; Ohta et al. 1993). In particular, the depolarizing influences via \(Cl_{Ca}\) channels could further lead to the activation of L-type Ca\(^{2+}\) channels and increased Ca\(^{2+}\) influx leading to increases in
smooth muscle contractility. In comparison, hyperpolarizing influences mediated via $K_{Ca}$ channels would be expected to decrease the smooth muscle mechanical activity by preventing opening of the $Ca^{2+}$ channels. Additionally, the ryanodine sensitive stores could be involved in setting up $Ca^{2+}$ oscillation and $Ca^{2+}$ waves (Komori et al. 1993; Missiaen et al. 1992).

Two of the most commonly studied pharmacological agents acting on the CICR are ryanodine and caffeine. Both are expected to stimulate the $Ca^{2+}$ release from SR in intestinal smooth muscle cells. In isolated intestinal myocytes from the guinea pig ileal longitudinal muscle (Dessy et al. 1996) and cultured smooth muscle cells derived from the human jejunum (Oh et al. 1997), ryanodine (10 $\mu$M) and caffeine (10 mM) increased cytosolic levels of $Ca^{2+}$ when measured with the $Ca^{2+}$ imaging methods. Ryanodine action is thought to occur by locking the SR release channels in an open state, and caffeine has been shown to sensitize the CICR mechanism to basal levels of $Ca^{2+}$ (Zucchi et al. 1997). Our objective was to study the effects of both activators of the CICR as well as of dantrolene, a blocker of the CICR (Zucchi et al. 1997), in the murine small intestine in order to reveal potential roles of the CICR in the regulation of the electrical activity as generated by smooth muscle cells in situ. Information was also obtained on the role of Cl' channels.
Methods

Preparation and recording of the electrical activities

A standard microelectrode technique, previously described in detail (Malysz et al. 1995; Malysz et al. 1996), was used to record slow waves from the murine small intestine. Adult mice used in this study were of either sex and supplied by Charles River Laboratories (CD1, St-Constant, Que.). The animals were cared for in accordance with the principles and guidelines of the Canadian Council on Animal Care.

All of the experiments described in this study were carried out in the presence of L-type Ca$^{2+}$ channel blockade with nifedipine, 3-5 μM. Previously, we have shown that the L-type Ca$^{2+}$ channel blockade abolishes action potential generation superimposed onto the plateau phases of the slow waves (Malysz et al. 1995; Malysz et al. 1996). In comparison, the slow wave activity is resistant to the treatment. Hence, the mechanisms of the regulation and generation of the slow wave can be studied as in this study in the presence of L-type Ca$^{2+}$ channel blockade.

Solutions and drugs

The composition of Krebs solution was (in mM): NaCl, 120.3; KCl, 5.9; CaCl$_2$, 2.5; MgCl$_2$, 1.2; NaHCO$_3$, 20.0; NaH$_2$PO$_4$, 1.2; and glucose, 11.5. The pH was 7.30–7.35. In experiments in which extracellular Cl$^{-}$ was reduced, 120.3 mM NaCl was substituted with 120.3 mM Na isethionate. Other chemicals used were nifedipine, ryanodine, caffeine, dantrolene, sodium isethionate, DIDS (4,4′-diisothiocyanato-
Figure 1. Summary of the effects of pharmacological treatments on the slow wave characteristics in the murine small intestine in the presence of L-type Ca$^{2+}$ blockade (nifedipine 3-5 μM). The filled black bars are controls and the dotted bars are in the presence of the experimental condition. The legends for the treatments with the n value stated in the brackets (n) are: 10 μM ryanodine (10 μM Ryan) (6), 50 μM ryanodine (50 μM Ryan) (7), 5 mM caffeine (5 mM Caff) (8), 1 mM caffeine (1 mM Caff) (7), 10 μM IBMX (6), 100 μM niflumic acid (7), 500 μM DIDS (6), 1 μM cromakalim (11), low extracellular chloride reduced by 90% (12), 2 mM TEA (8), 40 nM penitrem A (6), 2 mM 4-AP (6), and 30 μM dantrolene (7). * P < 0.05, ** P < 0.01.
stilbene-2,2'-disulfonic acid), niflumic acid, cromakalim, IBMX (3-isobutyl-1-methyl-xanthine), TEA (tetraethylammonium chloride), 4-AP (4-aminopyridine), and penitrem A. All except ryanodine (Calbiochem, La Jolla, Ca., USA) were purchased from Sigma Chemical Corp., St. Louis, Mo., USA.

Data analysis

Data were obtained usually from the same cells, before and in the presence of a drug. A difference was considered to be significant at P<0.05. Data are expressed as means ± SEM, with n being the number of different animals used. Paired or unpaired Student’s t tests, whenever appropriate, were employed for comparison of electrical activities determining the effects of drugs.

Results

Effects of CICR activation with caffeine and ryanodine

The most pronounced effect of 50 μM ryanodine and 5 mM caffeine, both expected to stimulate Ca^{2+} release from SR in intestinal smooth muscle (Dessy et al. 1996; Oh et al. 1997), was depolarization of the tissues by 8.7 ± 2.4 mV (P<0.02, n=7) and 8.8 ± 2.9 mV (P<0.02, n=8), respectively (figures 1 and 2). The effects of caffeine (5 mM) were temporally different than those of ryanodine. Following the addition of caffeine, cells transiently hyperpolarized (~2-5 mV) rapidly within ~1-3 minutes of perfusion. This transient phase lasted ~ 0.5-3 minutes and was followed by a depolarizing
Figure 2. Effect of CICR activation with caffeine and ryanodine on the electrical activity in the murine small intestine. (a) The first, top trace illustrates the control electrical activity prior to the addition of 5 mM caffeine. The second, third, and fourth traces show the electrical activities at 1, 3, and 10 minutes following the addition of caffeine (5 mM), respectively. Traces are from a continuous experiment. (b) The first, top trace depicts the control electrical activity prior to perfusion of ryanodine (50 μM). The second and third traces were obtained at 10 and 20 minutes following the addition of 50 μM ryanodine, respectively. Traces are from a continuous experiment. Throughout the experiments in (a) and (b), nifedipine (3-5 μM) was present.
phase, which was apparent by 10 minutes of perfusion. During this time, data for caffeine were collected and described in detail in figure 1. In contrast, ryanodine (50 μM) did not show any transient hyperpolarization, and the onset of depolarization was usually observed following 10-20 minutes of perfusion with this agent. Data with ryanodine as reported in figure 1 were collected 20-30 minutes following the addition of the agent. As reported in figure 1, ryanodine at 10 μM had no significant effects on the slow wave activity. In comparison, ryanodine at 50 μM and caffeine at 5 mM produced significant effects on the slow wave amplitude, average rate of rise of the upstroke, and frequency. In 8 experiments, 5 mM caffeine reduced the slow wave amplitude by 29.4 ± 9.3 % (P<0.03), the average rate of rise of the upstroke by 21.9 ± 6.9 % (P<0.05), the frequency by 33.6 ± 4.7 % (P<0.0001), and the duration by 30.2 ± 6.9 % (P<0.02). Ryanodine (50 μM) in 7 experiments reduced the slow wave amplitude by 54.2 ± 6.5 %, average rate of rise of the upstroke by 60.9 ± 8.9 % (P<0.003), and the frequency by 12.8 ± 2.0 % (P<0.01). Since the effects of caffeine and ryanodine on the slow wave properties were associated with depolarizations, we were concerned that the depolarizations per se with the agents were responsible for the observed results; therefore, the effects of high extracellular K⁺ on the slow wave were studied. We observed that increasing K⁺ by 2x and 3x depolarized the tissues by 6.0 ± 1.5 mV (n=7) and 13.6 ± 2.3 mV (n=7), respectively. Neither of these depolarizations were significantly different from those observed with ryanodine (50 μM) and caffeine (5 mM). Comparison of the effects of the high K⁺ solutions on the slow wave characteristics (amplitude, duration, rate of rise, frequency) with those of caffeine and ryanodine revealed that none were significantly
different. Hence, we could not rule out the possibility that the observed effects on the slow wave with ryanodine and caffeine were produced by the depolarizations per se and not by a direct effect of the agents on the slow wave properties.

As discussed above and indicated in figure 2, caffeine (5 mM) produced a transient hyperpolarization. In order to further elaborate on the mechanism responsible for the transient effect, additional experiments were carried out with a lower concentration of caffeine (1 mM) and IBMX (10 μM), a related compound to caffeine that shares the caffeine's blocking effect on cytosolic cyclic nucleotide phosphodiesterases. Both caffeine and IBMX consistently produced hyperpolarizations of 3.3 ± 1.3 mV (n=7, P<0.04) and 5.8 ± 1.5 mV (n=6, P<0.02), respectively. However, depolarizations were not observed. Furthermore, these agents affected none of the slow wave properties (figure 1). These experiments, therefore, indicate that the transient hyperpolarization observed with 5 mM caffeine was most likely due to its inhibitory effect on the cyclic nucleotide phosphodiesterases resulting in increases in cytosolic levels of cAMP and cGMP. This is also supported by experiments with forskolin and SNP, which are respectively known to increase cytosolic levels of cAMP and cGMP. Both, SNP and forskolin, in the murine small intestine produced hyperpolarizations (data not shown).
Role of chloride channels in the regulation of the slow wave and CICR induced depolarization

Since the activation of the CICR caused depolarization and a possible mechanism is activation of Cl⁻ channels either directly or indirectly by an increase in cytosolic Ca²⁺ levels, the effects of two classical Cl⁻ channel blockers, nitflumic acid (100 μM) and DIDS (500 μM) (Pusch et al. 1994; Large et al. 1996), and of the reduction of extracellular Cl⁻ were determined on the slow wave activity. In subsequent experiments, the effects of both the reduction of the extracellular Cl⁻ and the presence of the Cl⁻ channel blockers were tested on the extent of the depolarization produced with caffeine (5 mM).

Effects of chloride channel blockers on the slow wave

Addition of nitflumic acid and DIDS to the tissues resulted in hyperpolarizations of 9.8 ± 2.1 mV (n=7, P<0.005) and 3.4 ± 1.0 mV (n=6, P<0.03), respectively, during at least 5-10 minutes of perfusion of the blockers (figures 1 and 3). In addition, DIDS in 6 experiments also reduced the slow wave amplitude by 38.8 ± 6.8 % (P<0.003), the average rate of rise of the upstroke by 40.7 ± 10.1 % (P<0.03) and the duration by 25.0 ± 8.0 % (P<0.04). Nitflumic acid in 7 experiments also reduced the slow wave amplitude by 42.5 ± 9.1 % (P<0.005) and the average rate of rise of the upstroke by 42.6 ± 13.3 % (P<0.03). However, in contrast to the effects of DIDS, nitflumic acid decreased the slow wave frequency by 9.5 ± 3.4 % (P<0.04) and increased the duration by 26.6 ± 6.9 % (P<0.002). Since the above effects on the slow wave with the Cl⁻ channel blockers were
**Figure 3.** Effect of chloride channel blockade on the electrical activity in the murine small intestine. (a) The first, top trace depicts control electrical activity before the addition of niflumic acid. The second trace shows the effects of 50 μM niflumic acid and the third trace of 100 μM niflumic acid. Traces are from the same cell. There are ~10 minute lapses between the consecutive traces. (b) The first, top trace illustrates the electrical activity before the application of DIDS. The second trace was obtained ~20 minutes after the addition of DIDS (500 μM). The third and fourth traces show the effect of the addition of 5 mM caffeine in the presence of DIDS (500 μM) at respectively 2 and 10 minute intervals following the application of caffeine (5 mM). Traces are from a continuous experiment obtained from the same cell. Throughout the experiments in (a) and (b), nifedipine (3-5 μM) was present.
associated with significant hyperpolarizations, we considered that the hyperpolarization \textit{per se} produced the observed effects. For this purpose, we tested the effect of cromakalim, a K\textsubscript{ATP} channel opener, on the slow wave. As indicated in figure 1, 1 \( \mu \text{M} \) cromakalim hyperpolarized the tissues by \( 8.6 \pm 1.4 \text{ mV} \) (\( n=11, P<0.0002 \)), but it did not significantly affect any other slow wave characteristics. Hence, the effects of DIDS and niflumic acid on the slow wave parameters were unlikely due to their hyperpolarizing properties. In addition, it was observed that prolonged perfusion of the tissues with niflumic acid affected the tissue excitability; as a result, it became technically difficult to maintain stable impalements following 15-20 minutes of perfusion with the Cl\textsuperscript{-} channel blocker.

**Effect of reduction of extracellular chloride on the slow wave**

Previously, we have reported that following reduction of extracellular Cl\textsuperscript{-} by 90\% (120.3 mM NaCl substituted with 120 mM Na isethionate for a final extracellular Cl\textsuperscript{-} concentration of 13.3 mM in the modified Krebs solution), we could not identify any significant effects of the treatment on the slow wave properties (Malysz et al. 1995). However, non-significant mean reductions in the slow wave frequency and duration were observed as based on 6 experiments (Malysz et al. 1995). In the present study, we reevaluated the role of the reduction of the extracellular Cl\textsuperscript{-} by 90\% (i.e. 120.3 mM NaCl substituted with 120 mM Na isethionate). In 12 experiments, no significant effects on the resting membrane potential, amplitude, and the average rate of rise of the upstroke were observed (figures 1 and 4a). In comparison, reductions in the slow wave frequency by
12.7 ± 3.5 % (n=12, P<0.006) and duration by 21.5 ± 5. 3% (n=12, P<0.004) were identified.

**Effect of caffeine in the presence of low extracellular chloride**

Tissues were first treated with the reduced extracellular Cl⁻ solution as described above (i.e. in the presence of 13.3 mM extracellular Cl⁻ concentration) prior to the addition of 5 mM caffeine. The rationale for this series of experiments was that if CICR induced depolarization was predominantly mediated by the opening of Cl⁻ channels, then under the reduced extracellular Cl⁻ condition, caffeine would be expected to produce a stronger depolarization. This would occur since the reduction of extracellular Cl⁻ should favor greater efflux of the ion since the net Cl⁻ movement in gastrointestinal smooth muscle is in the outward direction (Casteels, 1969; Brading, 1971). As indicated in table 1 and figure 4b, caffeine (5 mM) under this condition did not produce significantly greater depolarizations when caffeine induced depolarizations in normal Cl⁻ (8.8 ± 2.9 mV, n=8) and reduced Cl⁻ (5.6 ± 2.4 mV, n=7) were compared (P=0.42). In addition, the caffeine induced depolarization obtained in the low extracellular Cl⁻ condition just failed to reach the statistical significance (n=7, P=0.06). Examination of the data revealed that in one influential experiment a hyperpolarization of 6 mV was observed. If data from this experiment were omitted in the analysis, then a significant depolarization of 7.5 ± 1.8 mV (n=6, P<0.009) was obtained. Again this value was not significantly different from that of caffeine in the normal extracellular Cl⁻ condition (P=0.72). Table 1 also reports other effects of caffeine (5 mM) on the slow wave properties in the presence of the reduced Cl⁻
Table 1. Effect of caffeine on the slow wave properties under different experimental conditions in the murine small intestine.

<table>
<thead>
<tr>
<th></th>
<th>Control (n=7) (in Low Chloride)</th>
<th>5 mM Caffeine (n=7) (in Low Chloride)</th>
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</thead>
<tbody>
<tr>
<td><strong>Resting membrane potential (mV)</strong></td>
<td>-61.9 ± 2.8</td>
<td>-56.3 ± 4.1 (^a)</td>
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<tr>
<td><strong>Amplitude (mV)</strong></td>
<td>20.9 ± 2.3</td>
<td>10.9 ± 0.7 (^c)</td>
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<tr>
<td><strong>Average rate of rise of upstroke (mV/s)</strong></td>
<td>138.2 ± 31.7</td>
<td>68.3 ± 14.4 (^b)</td>
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<tr>
<td><strong>Frequency (cpm)</strong></td>
<td>33.1 ± 2.6</td>
<td>22.4 ± 1.5 (^c)</td>
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<tr>
<td><strong>Duration (s)</strong></td>
<td>0.33 ± 0.03</td>
<td>0.28 ± 0.03</td>
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<table>
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<tr>
<th></th>
<th>Control (n=8) (in DIDS)</th>
<th>5 mM Caffeine (n=8) (in DIDS)</th>
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<tr>
<td><strong>Resting membrane potential (mV)</strong></td>
<td>-62.5 ± 3.6</td>
<td>-54.1 ± 4.0 (^c)</td>
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<td><strong>Amplitude (mV)</strong></td>
<td>11.5 ± 1.2</td>
<td>4.8 ± 1.1 (^c)</td>
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<tr>
<td><strong>Average rate of rise of upstroke (mV/s)</strong></td>
<td>38.7 ± 6.6</td>
<td>14.7 ± 3.1 (^c)</td>
</tr>
<tr>
<td><strong>Frequency (cpm)</strong></td>
<td>34.8 ± 3.0</td>
<td>21.0 ± 4.5 (^b)</td>
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<tr>
<td><strong>Duration (s)</strong></td>
<td>0.49 ± 0.05</td>
<td>0.43 ± 0.09</td>
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<thead>
<tr>
<th></th>
<th>Control (n=6)</th>
<th>5 mM Caffeine and 100 μM Niflumic acid (n=6)</th>
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<tbody>
<tr>
<td><strong>Resting membrane potential (mV)</strong></td>
<td>-60.0 ± 2.8</td>
<td>-54.6 ± 2.7 (^b)</td>
</tr>
<tr>
<td><strong>Amplitude (mV)</strong></td>
<td>13.6 ± 1.4</td>
<td>3.0 ± 1.4 (^c)</td>
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<tr>
<td><strong>Average rate of rise of upstroke (mV/s)</strong></td>
<td>55.8 ± 6.5</td>
<td>7.0 ± 4.8 (^c)</td>
</tr>
<tr>
<td><strong>Frequency (cpm)</strong></td>
<td>30.2 ± 4.3</td>
<td>16.8 ± 8.2 (^b)</td>
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<tr>
<td><strong>Duration (s)</strong></td>
<td>0.52 ± 0.04</td>
<td>0.32 ± 0.14</td>
</tr>
</tbody>
</table>

\(^a\) \(P=0.06\), \(^b\) \(P<0.05\), \(^c\) \(P<0.01\)
Figure 4. Effects of reduction of extracellular chloride and of caffeine in the presence of the reduced extracellular chloride on the electrical activity in the murine small intestine. (a) The top trace was obtained in normal extracellular chloride (133.6 mM). The lower trace shows the effect of reduction of extracellular chloride by 90% (extracellular chloride replaced with isethionate). Traces are from a continuous experiment with a 15 minute lapse between the two traces. (b) The three traces were recorded under the reduced extracellular chloride condition. The first, top trace is prior, the second trace 3 minutes after, and the third trace 10 minutes after the addition of 5 mM caffeine. All traces were recorded from the same cell. Throughout the experiments in (a) and (b), nifedipine (3-5 μM) was present.
solution. Neither of these effects were significantly different from those observed with caffeine (5 mM) in the normal Cl⁻ solution, hence, indicating that they might have been explained by the effect of caffeine on the resting membrane potential. In summary, experiments with caffeine in the presence of the reduced extracellular Cl⁻ solution do not support a predominant role of Cl⁻ channels in the CICR induced depolarization with caffeine.

**Effect of caffeine in the presence of chloride channel blockade**

Table 1 as well as figures 3(b) and 5 illustrate the effects of caffeine (5 mM) in the presence of Cl⁻ channel blockers. Since it became technically difficult to maintain stable impalements in the presence of niflumic acid for more than 15-20 minutes of perfusion, the effects of caffeine were determined in the tissues when it was added at the same time or up to 5 minutes following the addition of niflumic acid (100 μM). In contrast, in the presence of DIDS (500 μM) stable impalements were possible and caffeine (5 mM) was added following at least 20 minute pre-treatment with the blocker. As indicated in table 1, in the presence of the blockers, caffeine produced significant depolarizations. In the presence of DIDS and niflumic acid, the respective depolarizations were $8.4 \pm 1.1$ mV (n=8, P<0.0002) and $5.3 \pm 1.6$ mV (n=6, P<0.02). These depolarizations were not significantly different from those obtained with caffeine (5 mM) without the presence of the two Cl⁻ channel blockers. Other effects on the electrical slow wave with caffeine in the presence of the two blockers are reported in table 1. None of the observed effects were significantly different from those observed without the
Figure 5. Effect of caffeine in the presence of niflumic acid on the electrical activity in the murine small intestine. (a) The first, top trace illustrates the control electrical activity without niflumic acid and caffeine. The second, middle trace was obtained 2 minutes after and the third trace 9 minutes after the simultaneous co-application of niflumic acid (100 μM) and caffeine (5 mM). The three traces were obtained from a recording from the same cell. (b) The first, top trace depicts the control electrical activity without caffeine and niflumic acid. The second and the third traces were obtained in the presence of respectively 100 μM and 200 μM niflumic acid. There are 15 and 10 minute lapses between the consecutive traces from the top, respectively. The fourth trace was obtained 10 minutes after the application of 5 mM caffeine in the presence of 200 μM niflumic acid. All traces are from a continuous recording from the same cell. Throughout the experiments in (a) and (b), nifedipine (3-5 μM) was present.
blockers. In addition, figure 5(b) shows traces of an experiment in which it was possible to maintain a stable impalement in the presence of 200 μM niflumic acid. In this experiment, a depolarization of 12.6 mV was observed following the addition of 5 mM caffeine. Statistical analysis revealed a probability of 0.27 that this value belongs to the set of depolarizations produced with caffeine (5 mM) alone. Hence, one can not conclude that the depolarization of 12.6 mV is significantly different from that of caffeine alone. In summary, experiments evaluating the effects of caffeine in the presence of the Cl⁻ channel blockers do not support a dominant role for Cl⁻ channels in the caffeine or CICR induced depolarization. Hence, other possibilities had to be considered.

_Potential role of K_{Ca} channels in the regulation of slow wave and CICR induced depolarization_

Prolonged activation of CICR can lead to depletion of the ryanodine (and caffeine) sensitive stores (Iino et al. 1988). Since Ca^{2+} released from the ryanodine sensitive store has been linked to activation of K_{Ca} channels (Gagov et al. 1993; Mitra et al. 1985), depletion of the store could lead to the loss of activation of the channels and to subsequent depolarization since hyperpolarization mediated by K_{Ca} channels would be lost. However, TEA and penitrem A, both capable of blocking K_{Ca} channels in patch clamp studies on isolated myocytes from the murine small intestine (J.C.F. Lee and J.D. Huizinga, unpublished observation), had no effects on the slow wave parameters including the resting membrane potential (Figures 1, 6(a), and 6(b)). Hence, these data indicate that the K_{Ca} channels do not play a role in shaping the slow wave, pacemaker
Figure 6. Effect of K⁺ channel (K_{Ca} and K_{DR}) and of CICR blockade on the electrical activity in the murine small intestine. Top traces in (a), (b), (c), and (d) are controls. Lower traces in (a), (b), and (c) depict the effects of 2 mM TEA (a), 40 nM penitrem A (b), and 2 mM 4-AP (c). The lower trace in (d) shows the effect of 30 μM dantrolene. Top and lower traces for each panel (a)-(d) were obtained from continuous experiments; there are 10-15 minute lapses between the consecutive traces. Throughout the experiments in (a) - (d), nifedipine (3-5 μM) was present.
activity, nor do they play an important role in determining the resting membrane potential under conditions used in this study. The latter finding also argues against their involvement in mediation of the CICR induced depolarization.

Possible role $K_{DR}$ channels in mediation of CICR induced depolarization

Another type of $K^+$ channel that has been identified to be blocked by an increase in cytosolic $Ca^{2+}$ is the delayed rectifier, $K_{DR}$ (Post et al. 1995; Gagov et al. 1993). In this study, we examined the potential involvement of this channel by determining an effect of 4-AP, a blocker of certain types of $K_{DR}$ channels, on the electrical activity in the murine small intestine. As illustrated by figures 1 and 6(c), the addition of 4-AP (2 mM) caused depolarization of $5.6 \pm 1.6$ mV ($n=6, P<0.02$). Furthermore, reductions in the slow wave amplitude and the average rate of rise of the upstroke were observed by $18.4 \pm 6.1$ % ($n=6, P<0.05$) and $38.6 \pm 10.1$ % ($n=6, P<0.03$), respectively. We could not, however, exclude the possibility that the effects on the slow wave properties were due to depolarizations per se with 4-AP since comparable depolarizations obtained with high extracellular $K^+$ (9.2 mM) produced similar effects on the slow wave properties. There were no significant differences between the effects of the high $K^+$ and 4-AP on the resting membrane potential ($P=0.86$), the slow wave amplitude ($P=0.37$), and the average rate of rise of the upstroke ($P=0.63$).
**Effect of CICR blockade with dantrolene**

The role of CICR in the regulation of the slow wave activity was investigated by determining the effects of dantrolene, a blocker of CICR (Zucchi et al. 1997). The addition of dantrolene (30 μM) did not affect the resting membrane potential and the slow wave duration (figures 1 and 6(d)). In comparison, reductions in the slow wave amplitude (by 19.4 ± 3.3 %, P<0.003), the average rate of rise of the upstroke (by 32.5 ± 5.7 %, P<0.001), and the frequency (by 14.5 ± 3.2 %, P<0.006) were observed in 7 experiments. Hence, these experiments suggest a potential role of CICR in the regulation of the slow wave amplitude and frequency in the murine small intestinal musculature.

**Discussion**

The presence of Ca²⁺ induced Ca²⁺ release (CICR) is well established in many cell systems including intestinal smooth muscle cells. Herein, we report the first functional evidence of its role in the control of electrical activity of the intestinal musculature as measured from single cells in situ with microelectrodes. Blockade of CICR with dantrolene produced significant reductions in the slow wave frequency, amplitude and average rates of rise of the upstroke, but slow waves were not abolished. Hence, these data suggest that CICR is not directly responsible for the initiation of the electrical slow wave, but it may play a role in the amplification of the Ca²⁺ signal, which controls the slow wave frequency and amplitude. Our conclusion is consistent with a previous observation made by Publicover et al. (1992). While studying the intracellular Ca²⁺ oscillations in cultured canine colonic interstitial cells of Cajal (ICC), Publicover et al.
(1992) concluded that CICR may serve to amplify Ca\(^{2+}\) oscillations in the ICC. Since interstitial cells of Cajal are thought to be responsible for the initiation of the slow wave activity (Huizinga et al. 1997; Sanders, 1996), the effects of dantrolene may be explained by the action on ICC. A potential problem with dantrolene is that it is nonspecific. Blockade of plasmalemmal Ca\(^{2+}\) currents (Nasu et al. 1995) and NO synthase (Li et al. 1998) have also been reported with the CICR blocker. However, recent experiments in the murine jejunal tissue (Oh et al. 1997), in cultured human intestinal smooth muscle cells derived from the jejunum (Oh et al. 1997) and in isolated longitudinal smooth muscle cells of the rabbit small intestine (Kuemmerle et al. 1994) using comparable concentrations of dantrolene to those examined in our study were consistent with dantrolene blocking CICR. Additional studies with other known blockers of the CICR are needed to confirm the hypothesis that CICR plays a role in the amplification of Ca\(^{2+}\) signaling controlling the slow wave.

The most prominent effect of the activation of CICR judging by the effects of caffeine (5 mM) and ryanodine (30 \(\mu\)M) was depolarization of the intestinal smooth muscle with subsequent effects on the slow wave parameters due to the depolarization. Although our study did not provide a direct evidence for the stimulation of Ca\(^{2+}\) release by caffeine and ryanodine, recent studies in the guinea pig ileal longitudinal smooth muscle cells (Dessy et al. 1996) and on cultured smooth muscle cells derived from the human jejunum (Oh et al. 1997) showed that both agents, at comparable concentrations to those used in this study, increased cytosolic levels of Ca\(^{2+}\) when measured with Ca\(^{2+}\) imaging methods.
Previously, a patch clamp study in isolated rat longitudinal smooth muscle cells showed that caffeine activated an inward current with a rapid onset followed by a deactivation even in the continual presence of this agent (Ohta et al. 1993). Presumably, this current was activated by Ca$^{2+}$ release via the ryanodine receptors since the time course of the current matched the time course of the increase in intracellular Ca$^{2+}$ levels obtained with caffeine. Investigation of the ionic basis responsible for this inward current revealed that it was mediated through a Cl$^-$ conductance since the reversal potential of the current was most sensitive to changes in extracellular Cl$^-$ (Ohta et al. 1993). Our data on the murine small intestine do not support a predominant role of Cl$^-$ channels activated via Ca$^{2+}$ released from the ryanodine (or caffeine) Ca$^{2+}$ sensitive stores mediating the CICR induced depolarization. First, we showed that in the presence of the reduced extracellular Cl$^-$, the addition of caffeine did not produce a greater depolarization following ~10 minute perfusion with the agent as would be expected if Cl$^-$ channels were predominantly involved. In the intestinal smooth muscle cells, the net Cl$^-$ flow through the plasma membrane Cl$^-$ channels is in the outward direction (Brading, 1971; Casteels, 1969). Hence, the reduction of the extracellular Cl$^-$ should increase the driving force for Cl$^-$ efflux and produce a greater depolarization when stimulated with caffeine. Second, in the presence of Cl$^-$ channel blockade with DIDS or niflumic acid, ~10 minute perfusion with caffeine produced comparable depolarizations to those obtained without the Cl$^-$ channel blockers. Clearly, if Cl$^-$ channels were playing a predominant role, then attenuation of the CICR or caffeine induced depolarization would have occurred.
Our study further focused on the potential roles of K⁺ channels in the CICR induced depolarization. Two types of K⁺ channels were considered, the 4-AP sensitive delayed rectifier K⁺ (K_{DR}) channels and the Ca²⁺ activated K⁺ (K_{Ca}) channels. Support for the former is based on studies showing that ryanodine or H₁ receptor activation, which increases intracellular Ca²⁺ levels in coronary arterial smooth muscle cells (Gelband et al. 1993), and Ca²⁺ applied to the cytoplasmic surface of the K_{DR} in smooth muscle cells isolated from canine pulmonary arteries (Post et al. 1995; Gagov et al. 1993) produced significant reductions of the K_{DR} current. In contrast, the latter type of K⁺ channel (i.e. K_{Ca}) may have played an indirect role. It is possible that in our experiments the caffeine treatment led to a depletion of the intracellular ryanodine sensitive Ca²⁺ stores. Since Ca²⁺ release from the SR via ryanodine receptors has been linked to the activation of K_{Ca} channels in smooth muscle cells including gastrointestinal smooth muscle cells (Gagov et al. 1993; Mitra et al. 1985), it is possible that the depletion of the stores prevented further release of Ca²⁺ and subsequently the activation of K_{Ca} channels. The net effect would be depolarization since a hyperpolarizing influence mediated via K_{Ca} efflux would be lost. Although it was beyond the scope of this study with the available methodology to provide direct evidence for the role of K⁺ channels, the current approach could test whether the two types of K⁺ channels contributed to the maintenance of the resting membrane potential and were potentially involved in the CICR induced depolarization. In this study, we tested the effects of TEA and penitrem A, blockers of some types of K_{Ca} channels. Both in this tissue (i.e. murine small intestinal muscle strips) at concentrations identical to those used in this study increased the amplitude of phasic contractions (J.
Malysz and J.D. Huizinga, unpublished observation). Since TEA and penitrem A had no effects on the slow wave and on the resting membrane potential, we concluded that these channels were unlikely involved in the CICR induced depolarization. Similarly, no identifiable role for the $K_{Ca}$ channels could be deduced for the colonic slow waves (Carl et al. 1995). On the other hand, certain types of $K_{DR}$ channels might have been involved in the CICR induced depolarization. Perfusion of the tissues with 4-AP, a blocker of some types of $K_{DR}$ channels, led to marked depolarizations. Similarly, in gastric canine colonic musculature, 4-AP also produced depolarizations (Boev et al. 1985) indicating that these channels contribute to the resting membrane potential and are a potential target for the mediation of the CICR induced depolarization.

Nonselective cation or $I_{CRAC}$-like currents may play a role in the CICR induced depolarization. Some types of nonselective cation channels are activated by intracellular Ca$^{2+}$ (Conley, 1996), and, thus, they are possible targets for Ca$^{2+}$ released from the intracellular stores. Although caffeine was not reported to activate a nonselective cation current in the rat longitudinal smooth muscle cells (Ohta et al. 1993), in gastric smooth muscle cells of the toad Bufo marinus caffeine activated an 80-pS nonselective cation channel (Guerrero et al. 1994). Similarly, Ca$^{2+}$ activated or modulated nonselective cation channels have been identified in smooth muscle cells of the rat portal vein (Loirand et al. 1991), canine stomach (Sims, 1992), and the guinea pig small intestine (Pacaud et al. 1991; Inoue et al. 1990). The $I_{CRAC}$ or Ca$^{2+}$ release activated Ca$^{2+}$ current has been originally described in rat peritoneal mast cells (Hoth et al. 1992; 1993) and refers to a current activated by depletion of the intracellular Ca$^{2+}$ stores. Since similar
I_{CRAC}-like currents are also thought to be operative in smooth muscle cells (Fasolato et al. 1994; Low et al. 1993), a possible explanation for the caffeine and ryanodine induced depolarizations may be that the CICR activation resulted in depletion of the intracellular stores that in turn stimulated I_{CRAC}-like currents.

The main function of the Ca^{2+} induced Ca^{2+} release is through a positive feedback mechanism to increase cytosolic Ca^{2+} levels. In fact, the CICR induced depolarization that was observed in the murine small intestine may play a similar role. In this tissue, the depolarization would be expected to increase the rate of activation of the plasmalemmal voltage sensitive Ca^{2+} channels (i.e. L-type Ca^{2+} channels), hence, increasing the influx of Ca^{2+} leading to the subsequent increases in the cytosolic Ca^{2+} levels. Furthermore, the activated Ca^{2+} influx in response to the CICR induced depolarization may serve to replenish intracellular Ca^{2+} stores (Dessy et al. 1996). Our results also suggest another function of the CICR in the regulation of the slow wave although additional studies are still needed before this idea is to be accepted. The slow wave electrical activity plays an important role in the gut musculature. It restricts the action potential generation to the plateau phases, sets the (maximum) frequency of contractions, and determines the propagation characteristics of contractions (Szurszewski, 1987; Diamant et al. 1969). In the murine small intestine, a direct relationship has been shown among propagating electrical slow waves, propagating waves of intraluminal pressure, and pulsatile outflow of intestinal contents, thereby illustrating how slow waves control the peristaltic activity (Der-Silaphet et al. 1998). In \textit{W/W^*} mice, which lack the interstitial cells of Cajal
associated with the myenteric plexus and the electrical slow waves (Huizinga et al. 1995), abnormal peristaltic activity was observed (Der-Silaphet et al. 1998).

Since the nature of Cl⁻ channels in the murine small intestinal smooth muscle has not been established and no specific blockers are available, a role of Cl⁻ channels could only be assumed if several different approaches give consistent data. In the present study, the effects of the reduction of extracellular Cl⁻ and of two effective Cl⁻ channel blockers, DIDS and niflumic acid (Pusch et al. 1994; Large et al. 1996), which unfortunately also have other actions (Oba, 1997; Wang et al. 1997; Gribkoff et al. 1996; Liu et al. 1998; Spiro et al. 1985), were determined. The Cl⁻ channel blockers had variable actions on the slow wave frequency and duration, but with both reductions in the slow wave amplitude and average rate of rise of the upstroke as well as hyperpolarizations were observed. Hence, one may speculate that Cl⁻ channels contribute to the generation of the slow wave amplitude (plateau), but not to its initiation, as well as to the maintenance of the resting membrane potential. However, experiments with reduced extracellular Cl⁻ apparently argue against these roles of Cl⁻ channels. If they were involved, then increases in the slow wave amplitude and depolarization should have been observed when extracellular Cl⁻ was reduced since the net Cl⁻ flow is in the outward direction (Brading, 1971; Casteels, 1969). As illustrated in this study, there were no significant effects on the membrane potential or on the amplitude, but reductions in the slow wave duration and frequency were observed. Similar effects on the slow wave with various methods used to substitute for extracellular Cl⁻ were reported in the rabbit small intestine (El-Sharkawy et al. 1975). This suggests that these effects are a genuine outcome of the Cl⁻ reduction.
Caution, however, needs to be exercised when judging the effects of the reduction of extracellular Cl⁻. First, the lack of the effect on the amplitude may be explained by some physiological limit set for the maximal value for the slow wave amplitude. Second, smooth muscle cells contain plasmalemmal Cl⁻ transport carriers (e.g. Cl⁻ - HCO₃⁻ exchanger), and in response to the reduced extracellular Cl⁻, they may reset the intracellular Cl⁻ and driving forces for Cl⁻ movement to values different than those assumed. Hence, studies with more selective Cl⁻ channel blockers, when developed and available, will be needed to confirm the proposed roles of Cl⁻ channels in the regulation of the electrical activity. Our observation that Cl⁻ channels are not involved in the initiation of the slow wave is consistent with a recent study on isolated ICC. Thomsen et al. (1998) reported that ICC isolated from the murine small intestine exhibited spontaneous rhythmic inward current oscillations, which were not mediated via Cl⁻ channels but likely via nonselective cation channels. This contrasts with a recent study (Tokutomi et al. 1995) in which cells were isolated from the murine small intestine and labeled with anti kit antibody, a marker that predominately labels ICC in the musculature (Huizinga et al. 1995; Torihashi et al. 1995). The kit labeled cells were reported to exhibit spontaneous Cl⁻ current oscillations (Tokutomi et al. 1995). Hence, the authors proposed that the Cl⁻ oscillations were responsible for the initiation of the pacemaker electrical activity. In our opinion, insufficient data were reported showing that the studied cells were in fact interstitial cells of Cajal. In addition, anti kit- antibodies may also nonspecifically label other cell types, especially in isolation (T. L. Robinson, L. Faraway and J.D. Huizinga, unpublished observation).
In summary, we provide functional evidence utilizing a standard microelectrode technique for the presence of CICR mechanism in the murine small intestinal musculature. We showed that the activation of CICR induces depolarization, and the inhibition of CICR revealed that it might regulate the slow wave frequency and amplitude. The investigation of the ionic mechanisms of the CICR induced depolarization showed that it was unlikely to be mediated by $K_{Ca}$ or Cl' channels. Furthermore, evidence is provided illustrating that the slow wave initiation does not involve Cl' channels; however, Cl' channels may still be involved in the generation of the slow wave amplitude.
References


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Chapter Ten

General Discussion

Interstitial cells of Cajal (ICC) as pacemaker cells

Studies presented in Chapters Three through Six have a common theme. They illustrate a link between the absence of myenteric ICC and the lack of the slow wave activity. This finding supports Lars Thunerberg’s view that a subtype of ICC functions as pacemaker cells responsible for the generation of the slow wave activity (Thuneberg, 1982). To prove that ICC are the pacemaker cells, direct evidence was needed using isolated ICC. Recently, Thomsen et al. (1998) and Koh et al. (1998) cultured ICC from the murine small intestine and found regular inward current oscillations under the voltage clamp conditions. The oscillations had characteristics similar to the slow wave recorded in tissue; especially, they displayed insensitivity to the L-type Ca$^{2+}$ channel blockade. Studies in the near future on isolated ICC are still needed to further characterize the ionic mechanism(s) responsible for the pacemaker inward current oscillations including their pharmacological profile. In particular, the pharmacological sensitivity of the inward current should be the same as that of the slow wave. In this context, experiments in Chapters Seven and Nine provide an overview of the ionic dependency of the slow wave in the murine small intestinal musculature; this serves as an outline to which the work on isolated ICC should be compared.
Consequence of the lack of slow wave

Studies in this thesis (Chapters Three - Six) demonstrated that the musculature of the mutant mice lacking myenteric ICC and the slow wave activity was either electrically quiescent or showed action potentials in regular or irregular patterns. These action potentials were clearly distinguished from the slow waves by their shape and pharmacological sensitivity to the L-type Ca²⁺ channels blockers. Furthermore, in Chapters Four and Five, it was shown that the addition of TEA, a K⁺ channel blocker, to the mutant small intestinal musculature affected action potential generation leading often to the development of continuous spiking activity reaching very high frequency of up to ~70 cpm. In contrast, in control mice the same pharmacological treatment also enhanced the action potential generation, but in every case their generation was restricted to the plateaus of the slow waves. The continuous pattern of electrical activity, frequently observed in the mutant mice, was not recorded. Hence, the slow wave presence has a consequence on the pattern of action potential generation by the musculature.

Our laboratory recently compared the in vivo motor patterns of the proximal small intestine in $W/W'$ and in control mice after gavage of 0.5 mL of barium sulfate into the stomach (Der-Silaphet et al. 1998). In controls, the contrast fluid moved through the proximal small intestine in peristaltic waves ~47 times a minute propagating aborally at ~2 cm/s. In contrast, in $W/W'$ mice such an organized peristaltic motor pattern could not be observed, and back and forth, irregular movements of the intestine were observed. Although a net propulsion took place, it was much weaker than in controls. The reduced intestinal transit in $W/W'$ mice was also reported by others (Kamiya et al. 1985; Crowle,
Hence, the lack of the myenteric ICC and the absence of the slow wave were associated with the abnormal \textit{in vivo} motor patterns. This observation has an important clinical consequence. In several gastrointestinal disorders ICC were found abnormal (Crowle, 1983; Isezaki et al. 1997; Kenny et al. 1998a; Kenny et al. 1998b; Rumessen, 1996), thus, suggesting that abnormalities in the ICC and the slow wave may contribute to the motor pattern anomalies. Although the general acceptance of this notion requires further experimental support including studies on the affected human tissues, two recent studies utilizing animal models of inflammation provided evidence for this proposal. Inflammation in the distal canine colon, produced with mucosal exposure to ethanol and acetic acid (Lu et al. 1997), and in the proximal murine small intestine, obtained with \textit{Trichinella spiralis} (Der-Silaphet et al. 1996), affected ICC, especially their processes, the slow wave generation, and resulted in the abnormal \textit{in vivo} motor patterns.

\textbf{Search for selective markers of ICC}

A major obstacle in the study of ICC has been the lack of specific markers of these cells. Until recently, electron microscopy constituted the most reliable method for identifying ICC (see reviews by Thuneberg (1989) and Faussone-Pellegrini (1992)). In Chapters Three through Six, ICC were shown to be labeled with anti-kit antibodies using immunohistochemistry and with \textit{Kit} cRNA probes using \textit{in situ} hybridization. This labeling method has been now successfully utilized in the clinical characterization of gastrointestinal motor disorders. For example, the number of kit positive cells in the external muscle layers of the small intestine was shown to be 3\% of normal in two
patients with chronic idiopathic intestinal pseudo-obstruction (Isozaki et al. 1997). Similarly, in Hirschsprung’s disease, which is characterized by obstruction of the colonic transit and by the absence of intramural ganglia in the enteric neural plexuses in the affected areas, a very low density of kit immunostaining, presumably of ICC, was observed in the submuscular plexus area and within the circular muscle layer (Vanderwinden et al. 1996a). These and other clinical studies (Kenny et al. 1998a; Kenny et al. 1998b), although yielding interesting observations, do not directly show that ICC are missing in the affected areas. Additional morphological studies particularly with electron microscopy and physiological studies utilizing contractility and electrophysiological approaches are still needed to assess the consequences of the abnormal kit immunostaining.

Although we now have a method of labeling ICC, the need for other markers staining ICC is as great as ever. First, the kit immunohistochemical method does not appear to adequately label ICC in culture presumably due to the loss of the kit receptor expression on the surface of ICC during the isolation. Thus, the identification of another vital staining method for ICC is essential since it may not be possible to alter the isolation methods sufficiently to preserve the kit receptor and still obtain viable cells. Second, there still remains a possibility that the kit labeling method also stains other cell types in addition to ICC in the external muscular layers of the intestine. This is particularly important since in clinical studies the kit immunostaining is assumed to selectively label ICC. Third, a new selective method of staining ICC may provide an insight into an intrinsic function or role of ICC. In this context, the use of immunoreactive markers has
recently shown the presence of neuronal and endothelial NO synthase (Matini and Faussonne-Pellegrini, 1997; Xue et al. 1993) (although Vanderwinden et al. 1996a failed to confirm this) and heme oxygenase type 2 (Miller et al. 1998) in ICC. Furthermore, this suggests that ICC synthesize gaseous substances, which may be acting on neighboring cells including smooth muscle cells. However, neither of these staining methods are selective for ICC, but they also label other cell types in the external muscular layers (Matini and Faussonne-Pellegrini, 1997; Miller et al. 1998). In the opinion of the author, the single cell RT-PCR method will prove to be indispensable in the search of the unique markers for ICC. The RT-PCR technique has been already used successfully to confirm that the isolated ICC are positive for the Kit mRNA (Thomsen et al. 1998).

Role of the kit receptor-steel factor interaction

In Chapters Three through Six, ICC associated with the myenteric (or Auerbach plexus) were almost absent in \( W/W^v \) mice that express an abnormal kit tyrosine kinase receptor (Bernstein et al. 1990; Nocka et al. 1989). They were completely missing in \( S^{l}/S^{l}_d \) mice, which lack a membrane bound form of the steel factor (Williams et al. 1992; Besmer et al. 1993), and greatly reduced in numbers in \( W^{bd}/W^{bd} \) mice, which have a 2.8 Mb inversion of the DNA sequence upstream of the \( W/kit \) gene affecting the transcriptional activation of the Kit gene (Klüppel et al. 1997). Based on these findings, a hypothesis was formulated that the interaction between the steel factor and the kit receptor is required for both myenteric ICC and the pacemaker slow wave activity. Furthermore, data in Chapter Six that describe morphological staining of ICC provide an
insight into a possible role of the interaction. Methylene blue positive cells, presumably myenteric ICC, did not proliferate in $W^{bd}/W^{bd}$ mice between day 5 and 6 months after birth. In $+/+$ mice, in contrast, over the same period the number of ICC increased 11-fold in the small intestine. Hence, this study suggested that at birth ICC are normal, apparently developing without the need of the kit receptor-steel factor interaction, and the interaction appeared important over the next 6 months. Similarly, Bernex et al. (1996) concluded that kit was not required for migration, proliferation, or survival of myenteric ICC during the embryonic stages of development in the murine small intestine. Although these studies did not identify any morphological consequence for the steel factor-kit receptor interaction during the developmental stages, they do not necessarily disprove any functional role of the interaction during embryogenesis. Some kind of a function for the interaction is suggested by immunohistochemical and in situ hybridization studies illustrating the expression of the kit and steel factor markers in the embryonic gut in the proximity of the layer that will house mature myenteric ICC (Torihashi et al. 1996; Torihashi et al. 1995). Clearly, functional electrophysiological studies are needed to settle this controversy. One important finding, in this context, will be the identification of the type of electrical activity recorded in the musculature of newborn $W^{bd}/W^{bd}$ mice. As discussed above and in detail in Chapter Six, no differences in the numbers of ICC could be found between $W^{bd}/W^{bd}$ and $+/+$ mice shortly after birth although Kit mRNA could not be detected in the $W^{bd}$ mutant mice but readily observed in the wild type mice during embryogenesis. Hence, the lack of ability to record slow waves in the newborn $W^{bd}$
mutant mice would support the role of the kit receptor-stele factor interaction in the development of the pacemaker electrical activity during embryogenesis.

**Ionic basis of the slow wave components**

Chapters Seven through Nine of this thesis deal with the regulation of the electrical slow wave activity. In Chapter Seven, the ionic basis of the electrical slow wave in the murine small intestine was investigated. The data could be explained by a two component model for the slow wave similar to the one proposed by Tomita (1981) for antral slow waves. The two components are differentiated by their sensitivities to extracellular Ca\(^{2+}\) and Na\(^{+}\) (figure 1), the first or initial component being very sensitive to Ca\(^{2+}\) and the second component to Na\(^{+}\). Possible candidates involved in the first component could be either a low threshold T type-like Ca\(^{2+}\) channel or a Ca\(^{2+}\) activated (or augmented) nonselective cation channel permeable to Ca\(^{2+}\) and Na\(^{+}\) under the physiological conditions. So far, no conclusive evidence has been provided for either view. Identification of a rhythmic oscillation, likely mediated via a nonselective cation channel (Thomsen et al. 1998) in ICC of the murine small intestine, and dependency of the slow wave generation on intracellular Ca\(^{2+}\) (Chapter Eight) provide support for the latter view. On the other hand, it has been shown that isolated ICC or ICC-like cells from the canine colon (Lee and Sanders, 1993) and from the human small intestine (Rich et al. 1998) possess a low threshold voltage sensitive Ca\(^{2+}\) current. Thus, these observations provide support for the latter view. Future studies on tissues and on isolated ICC with
Figure 1. Proposed ionic components for the electrical slow wave in the murine small intestine. For clarification, the L-type Ca\(^{2+}\) channel component, which is not part of the slow wave, is also indicated. The slow wave consists of two components (1) non-L-type Ca\(^{2+}\) channel and (2) Na\(^{+}\) and Cl\(^{-}\) component. For the first component, two candidates are listed, see text for discussion.
specific blockers of nonselective cation channels and low threshold Ca\(^{2+}\) channels will provide valuable insights into the role of these channels in the slow wave generation.

The ionic basis of the second component is less clear although a Na\(^+\) conductance clearly plays an important role in its generation. This Na\(^+\) conductance does not involve TTX- or mexiletine-sensitive Na\(^+\) channels, but it may be mediated via a nonselective cation channel since nonspecific blockers of nonselective cation channels, Ni\(^{2+}\) and flufenamic acid, reduced the slow wave amplitude (Chapter Seven). Furthermore, the observation that phenylephrine increased the upstroke amplitude and reduced the plateau amplitude suggests that the second component likely consists of more than a single ionic component or channel (Chapter Eight). Whether Cl\(^-\) channels are also involved in the generation of the second component will require future studies by the use of selective Cl\(^-\) channel blockers. Unfortunately, at the present time these are not available. Studies in Chapter Nine with nonspecific Cl\(^-\) channel blockers, DIDS and niflumic acid, by showing a reduction in the slow wave amplitude provide evidence for their involvement. One conclusion, however, that can be drawn from these studies determining the effects of the Cl\(^-\) channel blockers is that Cl\(^-\) channels are not responsible for the slow wave initiation. This finding is supported by a study of Thomsen et al. (1998) but contradicts that of Tokutomi et al. (1995). In the latter study, kit immunopositive cells (not conclusively proven to be ICC) isolated from the murine small intestine exhibited rhythmic Cl\(^-\) oscillations. In contrast, in the former study on isolated ICC from the murine small intestine, the involvement of Cl\(^-\) channels in the generation of the pacemaker current
current rhythmic oscillations was excluded on the basis of the reversal potential for the pacemaker current (~10 mV compared to $E_{Cl}$ of ~70 mV in this study).

**Trigger component of the slow wave**

Previous studies in the small intestines of rabbits (Mills and Taylor, 1971; Cheung and Daniel, 1980) and cats (Specht and Bortoff, 1972; Specht, 1976) showed that slow waves can be triggered or entrained by extracellular stimulation. These findings support the voltage dependency of slow waves. Under the physiological conditions, the mechanism triggering the slow wave is thought not to be voltage sensitive since the slow wave initiation was only weakly voltage dependent, and under voltage clamp conditions, current oscillations could be recorded at an identical frequency to that of the slow wave (Connor et al. 1974; Tomita, 1981). Hence, some type of voltage independent mechanism is thought to trigger the slow wave oscillation, which also includes a voltage dependent component (Tomita, 1981).

An experiment described in figure 2 provides an insight into the identification of the trigger component of the slow wave. In the murine small intestine, small oscillations (~2-5 mV were sometimes observed at a depolarized resting membrane potential in the presence of cyclopiazonic acid and L-type Ca$^{2+}$ channel blockade. As illustrated in figure 2, hyperpolarization of the membrane potential with cromakalim, a K$_{ATP}$ channel activator, showed that at a critical threshold, the amplitude of the oscillation increased from the small value to ~10-15 mV. The very narrow range over which this occurred (~ at -57 to -60 mV) suggests the activation of a voltage sensitive ion channel. This
Figure 2. Identification of the trigger component for the slow wave generation in the murine small intestine. Trace (a) shows small amplitude oscillations prior to addition of 10 μM cromakalim, a $K_{ATP}$ channel activator. Traces (b) and (c) depict the effects of cromakalim (10 μM) at 10 seconds and 4 minutes of perfusion, respectively. Traces (d) and (e) were obtained following 15 and 18 minutes of washout of cromakalim, respectively. Traces (a) – (e) are from a continuous experiment from the same cell. In (a) – (e), 1 μM CPA and 3 μM nifedipine were present (J. Malysz and J. D. Huizinga, unpublished observation).
threshold voltage value is very similar to that reported to activate, below membrane potentials of -50 mV, a class of hyperpolarization gated nonselective cation channels. However, the insensitivity of the slow wave to milimolar concentrations of Cs⁺ (an effective blocker of this class of the nonselective cation channels) in the murine small intestine (J. Malysz and J.D. Huizinga, unpublished observation) and in the canine colon (Sanders, 1996) argues against the involvement of this class of channels in the slow wave generation. A recent preliminary report by Rich et al. (1998) on ICC-like cells isolated from the human small intestine provides another candidate, a low threshold Ca²⁺ channel. In their study, a voltage sensitive T-type like Ca²⁺ current could only be resolved at potentials below -50 mV (Rich et al. 1998). Hence, it is possible that the very small 2-5 mV oscillation functions as the trigger for the slow wave activating a T-type-like Ca²⁺ channel. Both of the electrical events, the trigger oscillation and the activated low threshold Ca²⁺ channel, are proposed to be essential for the generation of the full slow wave involving Na⁺ and potentially Cl⁻ conductances.

A nonselective cation channel activated by intracellular Ca²⁺ constitutes an ideal candidate for the trigger oscillations. This proposal also takes into account the effects of BAPTA/AM on the slow wave in the murine small intestine (see Chapter Eight). As shown, perfusion of the tissues with this intracellular Ca²⁺ chelator led to either abolition of the slow wave or marked reductions in the slow wave amplitude and frequency. Furthermore, nonselective cation channels may also be permeable to Ca²⁺ in addition to Na⁺ and K⁺ (Siemen, 1993). Thus, the abolition of slow wave under the Ca²⁺ free
conditions (Chapter Seven) may be explained by the involvement of a Ca\(^{2+}\) activated nonselective cation channel in the slow wave generation.

Although the proposed idea for the initiation of the slow wave by the trigger oscillation requires further experimental support, an unpublished observation on the canine colonic slow wave provides evidence for the presented model. In this study (J. D. Huizinga and L. Farraway, unpublished observation), an upstroke potential (slow wave resistant to L-type Ca\(^{2+}\) channel blockade) was found to be differentially sensitive to Ni\(^{2+}\) and Cd\(^{2+}\) during the hyperpolarizing current pulses. Ni\(^{2+}\) reduced the slow wave but a small oscillation remained, in contrast Cd\(^{2+}\) blocked both. In the context of the triggering component hypothesis, Cd\(^{2+}\) but not Ni\(^{2+}\) by blocking the trigger component completely prevented the slow wave initiation.

In summary, the model for the slow wave oscillation predicts sequential activation of the following ionic conductances: (1) Ca\(^{2+}\) or Na\(^{+}\) conductance via a Ca\(^{2+}\) activated nonselective cation channel generating of the trigger oscillation, (2) a Ca\(^{2+}\) conductance via a low threshold Ca\(^{2+}\) channel, and (3) Na\(^{+}\) and potentially Cl\(^{-}\) conductances. K\(^{+}\) channels would also be involved in determining the slow wave waveform; however, the penitrem A and TEA sensitive K\(_{Ca}\) channels can be excluded (see Chapter Nine). Future studies will establish the validity of this hypothesis. In the opinion of the author, an extremely useful method, which could provide valuable insights into the ionic basis of the slow wave, is the ion selective microelectrode technique. By measuring changes in the intracellular levels of Ca\(^{2+}\), Na\(^{+}\), Cl\(^{-}\) and K\(^{+}\) simultaneously with the slow waves, conductance changes may be linked to stages of the slow wave oscillation. This method
has already been successfully used in determining the ionic basis of cardiac action potentials (Kline, 1990).

**Regulation of the electrical activity by intracellular calcium stores**

Studies in Chapters Eight and Nine further characterize the importance of intracellular Ca\(^{2+}\) release mechanisms in the regulation of the electrical activity in the murine small intestine. Of the two types of Ca\(^{2+}\) release mechanisms examined, IP\(_3\) induced Ca\(^{2+}\) release (IICR) appears to be more important than Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) in the regulation of the slow wave. In particular, agents with different blocking actions on the IP\(_3\) signaling pathway culminating in Ca\(^{2+}\) release attenuated the slow wave generation and/or frequency, and agents with sensitizing or activating actions on the IICR increased the slow wave frequency. Similarly, agents, which stimulate IP\(_3\) synthesis, increased the slow wave frequency, in particular under the conditions where the frequency was artificially reduced with CPA (Chapter Eight). Furthermore, studies in Chapter Eight suggest separate mechanisms for the regulation of the slow wave frequency and amplitude since certain agents appeared to preferably attenuate the amplitude or frequency. The identity of these mechanisms still requires future studies, but these may be related to either differential sensitivities of the IICR in ICC and in smooth muscle or to the presence of two types of IICR in ICC.
Figure 3. Proposed model for the regulation of the slow wave by IP$_3$ induced Ca$^{2+}$ release. (a) Regulation of the slow wave by a single IP$_3$ induced Ca$^{2+}$ release mechanism based on Liu et al. (1995). (b) Regulation of the slow wave by two types of IP$_3$ induced Ca$^{2+}$ release mechanisms involving IP$_3$-A and IP$_3$-B receptors, see text for discussion.
Hypothesis for the slow wave generation and regulation by IP$_3$

Previously, Liu et al. (1995) proposed that the slow wave generation in the canine colon is controlled by IP$_3$. According to the model depicted in figure 3(a), the periodic release of Ca$^{2+}$ from the intracellular stores, which is sensitive to IP$_3$, activates the putative plasmalemmal pacemaker channel. Opening of this channel allows for influx of Ca$^{2+}$ or Na$^+$ and the generation of the pacemaker component subsequently triggering the slow wave oscillation. The reuptake of Ca$^{2+}$ into the intracellular stores involves the SR Ca$^{2+}$ ATPase pump. Thus, this explains the observed reductions in the slow wave frequency with CPA in the canine colon (Liu et al. 1995). All of these events are thought to occur in subplasmalemmal spaces, and they do not necessarily involve the whole cytosol (Liu et al. 1995). Data presented in Chapter Eight on the murine small intestine support this model, and they provide even a stronger evidence for the regulation of the slow wave as originally presented for the canine colon. Experiments described in detail in Chapter Eight, however, apparently suggest that two types of IP$_3$ sensitive release mechanisms are involved in the regulation of the slow wave, one responsible for the control of the frequency and the other for the regulation of the amplitude. A simplified model as outlined in figure 3(b) takes into account this speculation by identifying two types of IP$_3$ sensitive release mechanisms involving separate IP$_3$-A and IP$_3$-B receptors. The former is proposed to be responsible for the initial release of Ca$^{2+}$, the primarily function of which is to activate the IP$_3$-B release mechanism. This could occur either through the Ca$^{2+}$ activating property of the IP$_3$ receptor as suggested by lino (1990) or by the Ca$^{2+}$ induced synthesis of IP$_3$ by stimulating the Ca$^{2+}$ dependent PLC activity (Chien
and Cambier, 1990; Haeffner and Wittmann, 1992). Both of these pathways are indicated in figure 3(b). The release via the IP$_3$-B receptor is proposed to stimulate or augment the opening of the Ca$^{2+}$ dependent pacemaker channel. Similarly to the model by Liu et al. (1995), the reuptake of Ca$^{2+}$ into the SR involves the activity of the Ca$^{2+}$ ATPase pump; however, other reuptake mechanisms may also be involved. These proposed events result in the activation of the pacemaker channel, and they underlie the generation of the trigger component, which is sensitive to extracellular Ca$^{2+}$ and followed by the activation of ionic conductances involved in the generation of the full slow wave oscillation.

Clearly, the presented model (figure 3(b)) favors the notion that two types of IICR mechanisms are present in ICC. As explained above, this may not be necessarily the case since smooth muscle cells may determine the slow wave amplitude. Additional complexity, not indicated in this model, may be that the two types of release mechanisms (mediated by the putative IP$_3$-A and IP$_3$-B receptors) involve physically distinct intracellular Ca$^{2+}$ stores potentially refilled by different mechanisms. Future studies on isolated ICC, on smooth muscles, and on tissue will determine the validity of the proposed model.

Role of calcium induced calcium release (CICR)

The role of the ryanodine sensitive Ca$^{2+}$ stores in the regulation of the electrical activity in the murine small intestinal musculature is elaborated in detail in Chapter Nine. The main conclusions are that CICR does not appear to initiate slow waves, that it may
contribute to the amplitude and frequency generation, and that the activation of CICR leads to depolarization. The conclusion regarding its importance in the regulation of the slow wave requires further experiments with other known blockers of the CICR mechanism for this view to be generally accepted. Additionally, the identified CICR induced depolarization may be of physiological importance and determination of its ionic basis warrants future studies. Although the predominant role of Cl⁻ channels can be excluded, other possible candidates are the nonselective cation, I\textsubscript{CRAC}-like, and K\textsubscript{DR} channels (Chapter Nine).

Since the CICR mechanism has been linked to excitation-contraction coupling in skeletal and cardiac cells, the determination whether it plays a similar role in gastrointestinal smooth muscle cells should be the focus for future investigations. A combination of electrophysiological, Ca\textsuperscript{2+} imaging, and contractile studies, with objectives set out to determine the effects of agents known to block or stimulate the CICR mechanism, should determine whether there is a link among the Ca\textsuperscript{2+} influx, CICR, and the generation of the contractile activity.

**Intracellular calcium in smooth muscle cells and interstitial cells of Cajal**

Studies in Chapters Eight and Nine suggest that regulation of the slow wave generation (amplitude and frequency) involves intracellular Ca\textsuperscript{2+}, IICR, and to a lesser degree potentially CICR. One of the shortcomings of the utilized standard microelectrode technique in this thesis is the uncertainty as to whether the observed effects of pharmacological agents on the electrical activity were due to the actions on ICC, on
smooth muscle cells, or on both. The effects on the resting membrane potential, amplitude, average rate of rise of the upstroke, and the duration could be explained by the effects on both ICC and smooth muscle cells. Only the effect on the frequency can be attributed with any certainty to ICC. The limitation of the microelectrode method is easily overcome with the patch clamp methods allowing recording from isolated cells (ICC or smooth muscle cells). Hence, studies on isolated ICC and smooth muscle cells testing the effects of various pharmacological agents acting on IICR and CICR should be carried out. Furthermore, the proposed model for the slow wave initiation and generation suggests that the intracellular Ca^{2+} transients, which regulate the slow wave, are restricted to the subplasmalemmal regions, and they do not necessarily involve the whole cytosol. The support for or against this hypothesis will be provided one day with digital Ca^{2+} imaging methods. This method will also provide powerful insights into the control mechanisms of Ca^{2+} transients in ICC and smooth muscle cells.

**Mechanisms of the slow wave propagation in the external muscle layers**

Studies presented in Chapters Three through Six support the notion that in the small intestine the cellular origin of the electrical slow wave involves myenteric ICC. It still remains unclear how the pacemaker electrical activity generated by ICC spreads throughout the musculature into and throughout the circular and longitudinal muscle layers of the small intestine. Morphological studies show few gap junctions between ICC and smooth muscle cells at the level of the myenteric plexus (Thuneberg, 1989; Taylor et al. 1977). The gap junctions are, though, readily visible in the circular muscle layer
suggesting that electric propagation through the low resistance pathway may be a means by which the slow wave spreads throughout the circular muscle layer. Although gap junctions are not identifiable in the longitudinal muscle layer, this is likely related to the presence of gap junction of small size, which are not distinguishable by the electron microscopic methods. The support for this view is provided by an observation illustrating the spreading of neurobiotin, a tracer dye, within smooth muscle cells of the longitudinal muscle layer of the canine colon in which no gap junctions can be visualized (Farraway et al. 1995). The uncertainty remains for the role of smooth muscle cells in the slow wave propagation throughout the muscular muscle layers. Studies on the canine colon suggest that circular smooth muscle cells next to pacemaker ICC (associated with the submuscular plexus) possess an active mechanism for slow wave regeneration since the length constant along the layer decayed less than would be predicted if only passive membrane properties were involved (Liu and Huizinga, 1993). The active slow wave regeneration mechanism is also supported in this thesis (Chapters Four and Five) by showing that in some cases smooth muscle cells of W/W and SL/SLd mice, particularly in the presence of K\(^+\) channel blockade (0.5 mM Ba\(^{2+}\)), generated regular action potentials resembling slow waves. These, however, had a different ionic basis than slow waves since they were blocked with L-type Ca\(^{2+}\) blockers (nifedipine, verapamil, or D600). If some kind of active regeneration of the slow wave occurs within smooth muscle cells, then this finding would be clinically important since certain gastrointestinal motor disorders may be potentially caused at least in part by malfunctioning ICC and, hence, potentially by abnormal slow wave patterns (Isozaki et al. 1997; Vanderwinden et al.
1996a; Kenny et al. 1998a; Kenny et al. 1998b; Rumessen, 1996). It may be possible to design extracellular pacemakers similar to the heart pacemakers that control the electrical activity generation by smooth muscle cells and, thus, the peristaltic motor patterns. The finding that the slow waves can be triggered or entrained with extracellular field stimulation (Mills and Taylor, 1971; Inoue et al. 1989; Specht and Bortoff, 1972; Specht, 1976) supports attempts to develop such pacemakers. Furthermore, evidence for a potential role of ICC in gastrointestinal disorders has been now provided using experimental animal models of inflammation (Lu et al. 1997; Der-Silaphet et al. 1996).

Conclusions

*Cellular origin of the pacemaker slow wave activity in the small intestine involves myenteric interstitial cells of Cajal. The steel factor – kit receptor interaction is required for the presence of the network of myenteric ICC and the slow wave activity in adult animals.*

The data presented in Chapters Three – Six of this thesis illustrate that spontaneously genetic knock out mice with mutations affecting expression or structure of either the kit tyrosine kinase receptor (i.e. \( W/^{W^n} \) and \( W^{bd}/W^{bd} \) mice) or the steel factor (i.e. \( Si/Si^{pd} \) mice) lack both myenteric ICC and the pacemaker slow wave activity in the small intestine. Since no other cell type in the musculature appeared to be grossly affected by the mutations, it was concluded that the absence of the slow wave was most likely attributed to the lack of the network of ICC associated with the myenteric plexus.
In the absence of the slow wave, the mutant musculature was either electrically quiescent or generated action potentials in regular or irregular patterns. Furthermore, since the various mutations affecting the steel factor - kit receptor interaction produced the same result, it was concluded that this interaction is required for the presence of both the network of ICC and the slow wave activity in adult animals. In addition, evidence was provided in Chapter Six showing that this interaction is essential for the proliferation of ICC between day 15 and 6 months following birth during the growth of the external muscle layer.

*Generation of the slow wave involves Ca$^{2+}$ and Na$^+$ conductances. Cl$^-$ conductance is not involved in the initiation of the slow wave but may play a role in the regulation of the slow wave amplitude and of the resting membrane potential.*

Studies outlined in Chapter Seven illustrate that the slow wave amplitude is sensitive to extracellular Na$^+$ and Ca$^{2+}$ as well as to nonspecific blockers of nonselective cation channel blockers, Ni$^{2+}$ and flufenamic acid. Hence, this suggests that Na$^+$ and Ca$^{2+}$ conductances are involved in the slow wave generation. The involved conductances are distinct form TTX- and mexiletine- sensitive Na$^+$ channels, gadolinium sensitive nonselective cation channels and L-type Ca$^{2+}$ channels. In Chapter Nine, it was shown that nonspecific Cl$^-$ channel blockers, DIDS and niiflumic acid, reduced the slow wave amplitude and hyperpolarized the tissues, but they did not abolish the slow wave. These observations suggest that these channels are not involved in the initiation of the slow wave; however, they may still play a role in the regulation of the electrical activity.
Regulation of the slow wave (amplitude and frequency) involves IP$_3$ sensitive Ca$^{2+}$ release.

The data provided in Chapter Eight suggest that the periodic release of Ca$^{2+}$ from intracellular Ca$^{2+}$ stores, sensitive to IP$_3$, is involved in the initiation and regulation of the slow wave activity. This hypothesis is supported in this thesis by experiments determining the effects of pharmacological agents acting on the cytosolic Ca$^{2+}$ (BAPTA/AM), on the intracellular Ca$^{2+}$ store active reuptake mechanism (CPA), on IP$_3$ induced Ca$^{2+}$ release (2APB, thimerosal, caffeine), on PLC activity (neomycin and NCDC), and on agents, which stimulate IP$_3$ synthesis (carbachol, phenylephrine, and noradrenaline). These studies importantly identify an intracellular metabolic pathway that may potentially lead to the development of therapeutic approaches aimed at treating certain gastrointestinal motor disorders by modifying the slow wave frequency or amplitude.

Activation of Ca$^{2+}$ induced Ca$^{2+}$ release leads to depolarization. Ca$^{2+}$ induced Ca$^{2+}$ release may be involved in the regulation of the slow wave amplitude and frequency but unlikely in its initiation.

In Chapter Nine activation of CICR with caffeine and ryanodine produced depolarization. The CICR induced depolarization was not predominately mediated by Cl$^-$ channels nor likely by K$_{Ca}$ channels. A potential candidate mediating this depolarization is a class of K$_{DR}$ channels sensitive to 4-AP. Furthermore, since dantrolene, a blocker of
CICR, significantly reduced the slow wave amplitude and frequency, the CICR may play a role in the regulation of the slow wave. However, the involvement of CICR in the slow wave initiation may be excluded on the basis that this agent failed to completely abolish the slow waves.
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List of Publications

Published:


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- Presented at IX European Symposium on Neurogastroenterology and Motility, Maastricht, The Netherlands; October, 1998.

  John Malysz and Jan D. Huizinga. Control of action potential generation in the mouse small intestinal musculature by \( K_{Ca} \) and \( K_{DR} \) channels. Neurogastroenterol. Mot., 10, 461 (1998).


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- Presented at XV International Symposium on Gastrointestinal Motility; Rome, Italy; November, 1995.


- Presented at American Gastroenterological Association Digestive Disease Week; Boston, Mass., USA; May, 1993.


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