INTERSTITIAL CELLS: REGULATORS OF SMOOTH MUSCLE FUNCTION

Kenton M. Sanders, Sean M. Ward, and Sang Don Koh

Department of Physiology and Cell Biology, University of Nevada School of Medicine, Reno, Nevada

Sanders KM, Ward SM, Koh SD. Interstitial Cells: Regulators of Smooth Muscle Function. Physiol Rev 94: 859–907, 2014; doi:10.1152/physrev.00037.2013.—Smooth muscles are complex tissues containing a variety of cells in addition to muscle cells. Interstitial cells of mesenchymal origin interact with and form electrical connectivity with smooth muscle cells in many organs, and these cells provide important regulatory functions. For example, in the gastrointestinal tract, interstitial cells of Cajal (ICC) and PDGFRα+ cells have been described, in detail, and represent distinct classes of cells with unique ultrastructure, molecular phenotypes, and functions. Smooth muscle cells are electrically coupled to ICC and PDGFRα+ cells, forming an integrated unit called the SIP syncytium. SIP cells express a variety of receptors and ion channels, and conductance changes in any type of SIP cell affect the excitability and responses of the syncytium. SIP cells are known to provide pacemaker activity, propagation pathways for slow waves, transduction of inputs from motor neurons, and mechanosensitivity. Loss of interstitial cells has been associated with motor disorders of the gut. Interstitial cells are also found in a variety of other smooth muscles; however, in most cases, the physiological and pathophysiological roles for these cells have not been clearly defined. This review describes structural, functional, and molecular features of interstitial cells and discusses their contributions in determining the behaviors of smooth muscle tissues.

I. INTRODUCTION

“Interstitial cells” is a morphological term denoting a variety of cells of differing origins and phenotypes occupying spaces within the interstitium between the cells most prominent in defining a given tissue. In smooth muscle tissues fibroblasts, mast cells, macrophages, and interstitial cells of Cajal meet this definition. While mainly considered structural or immune cells by many morphologists, interstitial cells have come into prominence because they drive or contribute to the normal functions of smooth muscle organs, and remodeling or loss of these cells can lead to a variety of motor disorders. This review describes the physiology of the fibroblast-like classes of interstitial cells, which can include interstitial cells of Cajal (ICC), ICC-like cells, “Cajal-like” cells, fibroblast-like cells and telocytes in various anatomical descriptions of smooth muscle tissues (138, 213, 231, 292, 297, 322, 326, 342, 369, 389). There is a continuum of morphology in this group of cells, with some cells having abundant rough endoplasmic reticulum (ER), no basal lamina, no caveolae, and assuming a morphology attributed typically to fibroblasts. Other cells assume a more muscle-like appearance with less rough ER, but abundant smooth ER, prominent or even complete basal laminae, and caveolae (318, 376). Interstitial cells can form gap junctions with each other and with neighboring smooth muscle cells and can generate and conduct electrical signals that regulate smooth muscle excitability. Interstitial cells serve as pacemaker cells, propagation pathways for regenerative electrical events that cannot be propagated actively by smooth muscle cells, transducers of inputs from motor neurons, and stretch receptors. For historical reviews of the morphology and functions of interstitial cells, the reader is referred to a monograph by Lars Thuneberg (369) that reviews more than 200 morphological studies of muscle-like or fibroblast-like interstitial cells and a previous physiological review of electrical rhythmicity in visceral smooth muscles and role of ICC as pacemakers (326).

Work on interstitial cells in gastrointestinal (GI) muscles has dominated this field of investigation because there were important experimental opportunities that could be exploited. Interstitial cells in the tunica muscularis of GI muscles have distinct morphological features (98, 213, 369), mice with mutations in the protooncogene Kit have reduced populations of ICC in specific regions of the GI tract, and Kit mutants develop striking functional phenotypes (45, 167, 250, 378, 404, 417). Immunolabeling with antibodies against c-Kit has become a standard means for identification of “Cajal-like” cells (404) in a variety of organs. However, many studies of tissues outside the gut have encountered difficulties in labeling a distinct class of interstitial cells (other than mast cells) with this technique, and Kit mutants neither lacked the cells suspected to be inter-
stitial cells nor displayed functional defects. Thus progress in understanding the functions of interstitial cells in non-GI muscles has been slower. The discussion in this review will begin by reviewing highlights of research on ICC in the GI tract and then focus on progress made on this class of cells in other smooth muscle organs. Recent progress on a second class of interstitial cells, known for decades in the morphological literature as “fibroblast-like cells,” will also be discussed. These cells are labeled specifically in several smooth muscles by antibodies against platelet-derived growth factor receptor α (PDGFRα) (174, 207), and this has provided an important means of accessing these cells in physiological and genomic investigations.

II. ICC IN THE GASTROINTESTINAL TRACT

A. Structural Features of ICC and PDGFRα⁺ Cells and Networks

An important feature of GI interstitial cells (ICC and PDGFRα⁺ cells; see FIGURE 1 and TABLE 1 for clarification of the terminologies used to describe these cells and their specific anatomical localizations) is electrical coupling to smooth muscle cells. ICC and smooth muscle cells express a variety of gap junction proteins (68, 136, 337), but little is known about the nature and regulation of the gap junctions between interstitial cells and smooth muscle cells. Gap junctions facilitate communication between interstitial cells and smooth muscle cells (FIGURE 2). Electrical connectivity between interstitial cells and smooth muscle cells in GI muscles causes the tunica muscularis to behave, in fact, as a multicellular electrical syncytium. We have referred to this functional structure as the smooth muscle cell/ICC/PDGFRα⁺ cell (SIP) syncytium (209, 330; see FIGURE 3). Changes in electrical conductances in one type of SIP cell affect the electrical excitability of the other coupled cells. Spontaneous pacemaker activity generated by ICC conducts to smooth muscle cells and drives electrical slow waves and phasic contractions, and neural inputs to ICC and PDGFRα⁺ cells can conduct to smooth muscle cells and modulate contractile behavior. The integrated behavior of SIP cells defines what has been referred to classically as “myogenic” regulation of GI motor function. However, with current knowledge, the term myogenic is too limited to define a major division of GI regulation, and we suggest replacement of this term with SIPgenic to refer to the complex and integrated regulatory processes operating beneath the level of neural and hormonal regulation. It is now recognized that remodeling or

![FIGURE 1. A–C: c-Kit⁺ ICC-MY in mouse, monkey, and human gastric antrums, respectively. Note similarities in the structural organization of ICC in all three species. D–F: c-Kit⁺ ICC-MY (D, green) and PDGFRα⁺-MY (fibroblast-like) cells (E, red) are distinct populations of cells. These panels show cells from murine colon. Merged images from D and E are shown in F. G–I: intramuscular ICC (ICC-IM; G, c-Kit is green) in the monkey gastric fundus. ICC-IM form close associations with enteric motor nerve processes (nNOS⁺ motor neuron processes are shown in H, red). I: merged image of G and H. Scale bar in F applies to D–F, and scale bar I applies to G–I. (Authors are grateful to Dr. Masaaki Kurahashi for images in D–F and Dr. Peter Blair for images in B and G–I.)](https://www.physiology.org/journal/physrev/article/860)
loss of any component of the SIP syncytium or loss of connectivity between SIP cells can lead to abnormal motor behaviors in GI organs.

While the activity of the SIP syncytium is highly integrated in GI organs, knowledge about the functions of SIP cells has benefited from studies of specific cell types. Electrical coupling makes it very difficult to deduce the specific functions of one component in intact tissues. Even with mutant animals lacking specific elements of the SIP syncytium, it is still difficult to ascertain the specific functions of each cellular component. Dispersion of the tunica muscularis with proteolytic enzymes yields a mixture of cells. Smooth muscle cells are abundant and relatively easy to identify by their shapes, but interstitial cells are more rare and difficult to identify with certainty (typically amounting to <10% of cells within a given volume of muscle) and display morphologies that overlap with other cells in the nervous system. Generally found in larger animals including humans but not in lab rodents.

<table>
<thead>
<tr>
<th>Table 1. Nomenclature and localizations of interstitial cells in the tunica muscularis of the GI tract</th>
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<tr>
<td><strong>Organ</strong></td>
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<td>Esophagus</td>
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ICC and PDGFRα⁺ cells are as observed by c-Kit and PDGFRα⁺ immunoreactivity, respectively. Presence and relative abundance are denoted by + or −, respectively. References are representative examples, not an exhaustive listing; see text for further details. ICC-MY are from a network of cells between the circular and longitudinal muscle layer and have a pacemaker role in stomach, small bowel, and colon. ICC-MY have been called ICC-AP or ICC-MF by some authors (see text); however, these terms are misleading because ICC-MY are not physically within or a component of the myenteric (Auerbach’s) plexus. ICC-MY are spindle-shaped intramuscular cells lying in muscle bundles between muscle cells and in close apposition to varicose processes of enteric motor neurons. ICC-IM have also been called ICC-CM or ICC-LM to designate the muscle layer in which the ICC-IM were observed. ICC-IM are referred to as ICC-DMP in small intestine due to the clustering of these cells among the nerve process of the deep muscular plexus. Like ICC-IM, the ICC-DMP are closely associated with varicosities of enteric motor neurons. ICC-SM lie along the submucosal surface of the circular muscle layer. These have a pacemaker function in the colon. ICC-SS lie at the subserosa of the colon. *Species-dependent distributions: ± presence, absence, or low abundance depending on species. Generally found in larger animals including humans but not in lab rodents.
it again becomes problematic to maintain voltage control in voltage-clamp studies or to know which cell has generated a particular response. Another serious problem is that ionic conductances that define the functions of SIP synecytium cells are lost in cell cultures (436). Therefore, it is difficult to see the value of cell cultures for studies of the native behaviors of SIP cells.

Genetically engineered mice with cell-specific expression of fluorescent reporters have become important tools for studies of SIP cells (221, 311, 419, 436). SIP cells from reporter strains of animals can be identified unequivocally for electrophysiological and imaging studies, and fluorescent activated cell sorting (FACS) cells can be used to collect purified populations of cells for comprehensive sequencing of transcriptomes. Expression of inducible Cre recombinase in specific SIP cells has been accomplished and provides a powerful new technique for future studies of interstitial cells (131, 200).

Another suggestive anatomical feature of interstitial cells is their close associations with nerve varicosities, identified originally by electron microscopy (160, 318, 397). In the case of ICC, this relationship was noted by Santiago Ramón y Cajal (47), and he suggested that ICC were a type of peripheral neuron that connects motor neurons to local effector cells, such as smooth muscle cells. We know now that ICC are not neurons and of mesodermal origin (238, 379, 409, 430). Close associations between neurons and ICC were subjects of note in many classical electron microscopic examinations of these cells (73, 98, 179, 318). PDGFRα⁺ cells are also closely associated with terminals of neurons (36, 221, 434); however, the very close areas of apposition (<20 nm) between nerve varicosities and ICC described in many species have not been described for PDGFRα⁺ cells. Close associations between intramuscular ICC (ICC-IM) are relatively easy to identify in transmission electron micrographs in experimental animals and human GI muscles and have been described as “synapse-like.” Close contacts might indicate innervation of ICC by motor neurons. If neurotransmitters are released into regions of close association, very high concentrations might be achieved in the tiny postjunctional volumes, and transduction of neural inputs may tend to be focused at the sites of close contact. High junctional concentrations might also increase the rates of metabolism and/or reuptake of neurotransmitters and metabolites.

Close connections have also been described between nerve varicosities and smooth muscle cells (73, 264, 397), but these junctions appear either to be more rare or, at least, have been deemphasized or gone unmentioned in many morphological reports. The physical proximity of motor neurons to interstitial cells led to much speculation about the role of these cells in transducing neural inputs; however, functional innervation is much more controversial and difficult to demonstrate (see sect. IIF).

ICC have an abundance of mitochondria, moderately well-developed Golgi, caveolae, and rough and smooth ER. The perinuclear region is often densely packed with mitochondria and cisternae of ER running along the plasma membrane. The spaces between ER and the plasma membrane create tiny volumes in which localized Ca²⁺ transients may regulate plasma membrane ion channels and other functions of interstitial cells, such as pacemaker activity and responses to neurotransmitters. ICC have caveolae and a basal lamina in many regions of the gut, but these features are typically lacking in PDGFRα⁺ cells. Microtubules and thin and intermediate filaments are observed in ICC, but myosin thick filaments are not often found (320). Another difference between ICC and PDGFRα⁺ cells is that the latter are typically less electron dense than ICC within the same thin sections. A great abundance of rough ER is commonly found in PDGFRα⁺ cells compared with ICC, and this feature was considered “fibroblast-like” by electron microscopists. As mentioned previously, gap junctions are formed between ICC and PDGFRα⁺ cells and smooth muscle cells (158, 159). While PDGFRα⁺ cells tend to display similar ultrastructure features in various organs and in different species, ICC display a variety of structural and ultrastructural characteristics between species and organs, particularly in the abundance of caveolae and completeness and prominence of their basal laminae (211). However, there are not clear differences in ultrastructure that can be extrapolated to all regions. Some investigators have described ICC as specialized smooth muscle cells (88, 98, 179, 376). For example, ICC in the deep muscular plexus of the small intestine express α enteric actin, an actin isoform associated with GI smooth muscle cells (334), smooth muscle myosin light chains, and sparse distribution of thick filaments (376). In other studies, ICC are described as more...
fibroblast-like (66, 113, 212, 309, 314). Cells more like smooth muscle have more complete basal laminae and prominent caveolae; these features are more sparse or lacking in cells with a fibroblast-like appearance.

Most studies of ICC have been performed on mammalian species and limited primarily to common laboratory animals and humans. Recent, morphological reports describe ICC in the GI tract of zebrafish (14). The morphological features of these cells are similar in description to their mammalian counterpart; however, caveolae were not observed in either ICC or smooth muscle cells. ICC in zebrafish can also be identified by antibodies for c-Kit.

Ultrastructural characteristics are useful for identification of ICC and PDGFRα+ cells, but the technical demands of transmission electron microscopy (TEM) make this technique suboptimal for efficient characterizations of interstitial cell distributions and clinical assessments of the state of interstitial cells in human muscles (where frequently adequate fixation to preserve identifying features is difficult to achieve).

1. Histological and immunomarkers for ICC

For many years interstitial cells were difficult to investigate because these cells represent only a fraction of the total cells.
in the tunica muscularis, and there were no specific labels for these cells. Several histological stains were used to highlight ICC, such as methylene blue (366, 369), Champy-Maillet zinc iodide-osmic acid (59, 206, 321), Golgi (silver impregnation) staining (47, 206), rhodamine 123 (402), and NADH diaphorase (95, 420). None of these techniques was highly specific for interstitial cells, and successful staining was accomplished in only some regions of the gut and in only certain species (315). These limitations led one author to summarize various histological methods for identifying interstitial cells as “capricious” (58). For many years, the most reliable method for identification of interstitial cells was TEM, and a major obstacle in morphological studies was the inability to confirm that cells stained by histological techniques and imaged with light microscopy were the same cells identified by ultrastructural criteria with TEM. The ability to image cells with light and electron microscopy was an advantage of the NADH diaphorase technique (420). A study of the dog small intestine explored the nature and abundance of intermediate filaments in ICC within the deep muscular plexus (ICC-DMP) and discovered that vimentin filaments were predominant (376). Vimentin became the first immunolabel for interstitial cells and was used widely in many organs to identify populations of these cells. However, vimentin may not distinguish between c-Kit$^{+}$ (ICC) and PDGFR$^a$ cells.

Investigators studying hematopoietic cells and developmental defects in melanocyte and germ cells in Kit (White spotting; W) mutants (49, 118) observed that cells in a variety of tissues express Kit. To study the functions of c-Kit more broadly, neutralizing antibodies (ACK2) were administered to neonatal mice (250). These studies found that a population of cells within the small intestine expressed c-Kit-like immunoreactivity, and neutralizing c-Kit antibody injections produced severe defects in intestinal motility described as paralytic ileus. Closer inspection showed that ileal muscles lacked normal phasic contractile activity, but instead displayed clusters of arrhythmic contractions. Responses to agonists (bradykinin and acetylcholine) were also altered. Abnormal contractile patterns were also observed in ileal muscles of W/W$^v$ mice. Major morphological defects were not apparent in enteric nerves or smooth muscle cells of ACK2-treated mice. Immunolabeling demonstrated c-Kit$^+$ cells in the region surrounding the myenteric plexus and within the circular muscle layer. Loss of mechanical rhythmicity and reduction in c-Kit$^+$ cells after treatment with neutralizing antibodies suggested that the cells expressing c-Kit might be ICC. However, technical difficulties, including the inability to utilize the antibodies for immunocytochemistry or to identify the immunolabeled cells as ICC with other histological techniques, made it impossible for these authors to identify the c-Kit$^+$ cells as ICC definitively (250).

Positive identification of c-Kit$^+$ cells in the GI tract was eventually revealed by colabeling cells with methylene blue and by immunocytochemistry (378). Examples of ICC from mouse, monkey, and human gastric antral muscles, labeled with antibodies to c-Kit, are shown in Figure 1, A–C. Treatment of newborn mice with neutralizing c-Kit antibodies reduced or abolished the number of c-Kit immunopositive cells, the number of cells labeled with methylene blue, and cells with ultrastructural features of ICC (378). Additional studies showed that the cells in the region of the myenteric plexus (i.e., in the space between the circular and longitudinal muscle layers) that labeled with c-Kit antibodies (ICC-MY) were reduced in intestinal muscles of W/W$^v$ mice (167, 404). Mast cells are also labeled with c-Kit antibodies. However, few mast cells are present in the tunica muscularis of mice kept in clean animal facilities. Labeling with c-Kit antibodies became the standard technique for study and identification of ICC, and this technique has been widely exploited in developmental studies, pathological evaluations, and molecular and physiological studies during the past two decades. Much progress in our knowledge of ICC has occurred due to studies made possible by the use of c-Kit antibodies. c-Kit labeling was also adopted for diagnosis of gastrointestinal stromal tumors (GISTs) after GISTs were found to express c-Kit and may develop from ICC due to gain-of-function mutations (discussed further in sect. IIJ) (149).

Progress on ICC has depended on development or novel application of technology and reagents (Table 2). When c-Kit antibodies directed at extracellular epitopes were found to label ICC, we tried immunolabeling of live cells after enzymatic dispersion. However, the proteolytic enzymes used to disperse cells appear to damage the extracellular epitopes. Thus it became desirable to develop reporter strains with constitutive expression of fluorescent reporters. Unfortunately, the cell-specific promoters that drive expression of Kit in ICC are unknown, so it was not possible to generate a reporter strain with a simple knock-in strategy. The first strain developed utilized endogenous Kit promoters with a novel green fluorescent protein, ZsGreen, knocked into the first exon of the Kit gene, creating a null Kit allele, W(ZsGreen)$^{+/+}$ (418). Heterozygous (W[ZsGreen]$^{+/+}$) had tiny fluorescent dots in Kit expressing cells. This was an exciting advance, but the expression of ZsGreen was low in most ICC. Pacemaker ICC expressed only a few green dots per cell. Another approach utilized a similar strategy to exploit the endogenous cell-specific promoter and express a bright green fluorescent protein (copGFP) as the knock-in reporter (311, 436). Cells of Kit$^{copGFP/+}$ mice were highly fluorescent and visible in intact muscles and in cell dispersions. It should be noted that cells can even be visualized readily in living Kit$^{copGFP/+}$ mice using a fluorescence micro-endoscope inserted into the abdomen (unpublished observations). The reporter strain and cells dispersed from the tunica muscularis have been used in many subsequent physiological and molecular studies (discussed in sections below) and offered, for the first time, an efficient means of isolating and purifying living ICC.
Despite the progress made with c-Kit immunohistochemistry, the search for molecular reagents to identify and manipulate ICC populations goes on. A striking advance occurred when antibodies developed from GIST tumors (discovered on GIST-1; DOG-1) were found to label GIST tumor cells and ICC (93, 415). It was later discovered that DOG-1 corresponded to the gene product of \( \text{ANO1} \) (aka \( \text{TMEM16a} \)). A gene array screen of transcripts expressed by small intestinal ICC showed \( \text{Ano1} \) to be one of the most highly expressed genes in ICC (52), and the gene product of \( \text{Ano1} \), a chloride channel known as ANO1, is expressed robustly and exclusively by ICC throughout the GI tract (35, 122, 172). An advantage of labeling ICC in GI muscles with ANO1 antibodies is that mast cells are not labeled, and therefore, this method may be superior to c-Kit labeling in conditions in which mast cells infiltrate the muscle layers or, as in human GI muscles, where they are often abundant.

2. Histological and immunomarkers for PDGFR\( \alpha^+ \) cells

PDGFR\( \alpha^+ \) cells were referred to as fibroblasts or “fibroblast-like cells” (FLC) by morphologists (213). While TEM provided a description of the ultrastructural features of FLC and close appositions made by these cells to other cellular constituents of the muscularis, it was difficult to get a full appreciation of the extent and distribution of these cells throughout the GI tract, the major anatomical niches they occupy, and the relationships of these cells to other cells from the limited number of images in the published literature. Histological techniques were unable

### Table 2. Milestones in interstitial cell research in the GI tract

<table>
<thead>
<tr>
<th>Year(s)</th>
<th>Major Investigation and Advances</th>
<th>Key Reference Nos.</th>
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<tbody>
<tr>
<td>Before 1985</td>
<td>Morphological studies of ICC; controversies with staining techniques; EM considered the &quot;gold standard&quot; for identifying ICC.</td>
<td>47, 98, 113, 179, 309, 314, 369, 434, 435</td>
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<td>1980-1994</td>
<td>Whole muscle experiments; dissection to remove areas rich in specific ICC populations; use of &quot;selective&quot; poisons to destroy ICC function.</td>
<td>17, 29, 90, 137, 247, 354, 355, 370, 402</td>
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<td>1989</td>
<td>Isolation of fresh ICC and identification of cells by morphological features; demonstration of intrinsic electrical rhythmicity in isolated ICC.</td>
<td>235</td>
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<td>1992–present</td>
<td>Use of c-Kit mutants and c-Kit neutralization to study physiological functions of ICC.</td>
<td>45, 167, 250, 377, 378, 400, 403, 404</td>
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<tr>
<td>1994–present</td>
<td>Recognition of Kit immunohistochemistry as specific means to identify and characterize ICC networks.</td>
<td>44, 45, 167, 378, 404</td>
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<tr>
<td>1994–present</td>
<td>Demonstration of the role of ICC in electrical rhythmicity.</td>
<td>44, 45, 82, 125, 167, 279, 378, 404</td>
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<tr>
<td>1996–present</td>
<td>Demonstration of the role of ICC in motor neurotransmission.</td>
<td>22, 24, 45, 178, 324, 400, 408</td>
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<td>1996–present</td>
<td>Association of ICC loss or growth with pathophysiological conditions.</td>
<td>205, 249, 252, 279, 282</td>
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<td>1998</td>
<td>Discovery that c-Kit is a marker for gastrointestinal stromal tumors (GIST); GIST may arise from gain-of-function mutation in c-Kit of ICC.</td>
<td>78, 145, 149, 151, 269</td>
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<td>1998–2009</td>
<td>Culturing of ICC for more detailed physiological studies.</td>
<td>208, 368, 410</td>
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<td>2004–present</td>
<td>Use of flow cytometry to quantify and FACS to purify immunolabeled ICC.</td>
<td>52, 53, 161, 276, 277</td>
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<td>2004–present</td>
<td>Following activation of interstitial cells and propagation of pacemaker activity with digital imaging of ( \text{Ca}^{2+} ) transients.</td>
<td>146, 241–243, 283, 423</td>
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<td>2005</td>
<td>Demonstration of the role of ICC as stretch receptors.</td>
<td>214, 417</td>
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<td>2008</td>
<td>Isolation of progenitor cells capable of generating ICC.</td>
<td>16, 248</td>
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<td>2009</td>
<td>Reporter strains of mice in which freshly dispersed interstitial cells can be isolated and positively identified in mixed populations of cells.</td>
<td>221, 311, 418, 436</td>
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<td>2007-2009</td>
<td>Recognition of the importance of Ano1 (encoded by ( \text{Tmem16a} )) in ICC and its role in pacemaking.</td>
<td>52, 122, 172, 436</td>
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<td>2009</td>
<td>Identification of PDGFR( \alpha ) as a specific marker for fibroblast-like cells.</td>
<td>174, 177</td>
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<td>2011</td>
<td>Isolation of PDGFR( \alpha^+ ) cells using a reporter strain and physiological studies suggesting their role in inhibitory neurotransmission.</td>
<td>221</td>
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<tr>
<td>2013</td>
<td>Cell-specific iCRE strains that allow inducible knockdowns of genes/proteins in interstitial cells.</td>
<td>131, 200</td>
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References are representative examples to help the reader into the literature and not meant to be an exhaustive listing; see text for further details.
to identify FLC specifically, and few studies considered a role for these cells in regulating the behavior of the smooth muscle tissues.

Two immunomarkers were initially found to label FLC. The discovery that many GIST express c-Kit (149) was accompanied by reports that GIST cells and ICC also express CD34 (312). Others reported that CD34+ cells were adjacent to ICC, but represented a distinct population of cells (289, 387, 388). Cells with CD34-like immunoreactivity in the human small GI tract were identified as c-Kit+ FLC. Thus CD34 became a first immunolabel for FLC, but it was also recognized that CD34 is not a specific label in the gut wall, and other mesenchymal cell types express this antigen (289, 387). The guinea pig GI tract was found to express small-conductance Ca2+-activated K+ channels (SK2 and SK3), and SK3+ cells were found to be c-Kit− cells in the myenteric region and within the circular muscle layers of GI muscles (202). Two additional studies reported labeling of c-Kit− cells with antibodies to SK3 in human and mouse GI muscles (108, 386). SK2 expression was dominant in smooth muscle cells, but transcripts of SK3 were also found in these cells (310). These data are consistent with immunohistochemical findings showing punctate SK3 immunoreactivity in smooth muscle cells (202). Thus labeling with antibodies to SK3 is also nonspecific under some circumstances.

Significant progress occurred when FLC throughout the GI tracts of adult animals were found to express PDGFRα (174, 177). PDGFRα+ cells were shown to be FLC by immunocytochemistry and robust expression of SK3. PDGFRα+ cells are distinct from c-Kit+ ICC and unaffected in Kit mutants in which ICC are greatly reduced (FIGURE 1, D–I). PDGFRα+ cells form networks adjacent to ICC in the region of the myenteric plexus and are intertwined with ICC and processes of enteric neurons in muscle layers (174, 221). Similar distributions of PDGFRα+ cells and interrelationships with ICC and enteric motor neurons have been observed in several mammalian species including humans (35, 36, 50, 220, 221). Thus PDGFRα antibodies have become a reliable marker for FLC, and most investigators have adopted the terminology of PDGFRα+ cells to refer to these cells. As more is learned about the phenotype (chemical coding) of this class of interstitial cells, more specific terminology may become advantageous, because PDGFRα is expressed by many cells in the body and at various stages of development. At the present time, specific markers to distinguish cells within the region of the myenteric plexus (PDGFRα+−MY; FIGURE 1, D–F) or within the muscle bundles of the circular and longitudinal muscle layers (PDGFRα+−IM; FIGURE 1, G–I) have not been identified. Developing specific labels for subclasses of PDGFRα+ cells will allow finer resolution of their phenotypes and functions in the future.

In addition to the obvious advantages of a robust and selective immunolabel for morphological studies and pathological evaluations of PDGFRα+ cells, a mouse engineered to express a histone 2B-eGFP fusion protein driven off the endogenous promoter for PDGFRα (135) was found to have bright eGFP fluorescence in nuclei of cells immunoactive for PDGFRα antibodies (221). Cells with eGFP recapitulated the distribution of PDGFRα+ cells in the gut with high fidelity. Constitutive labeling of PDGFRα+ cells opened the door to molecular phenotyping and physiological studies of these cells (as discussed below).

B. Notes on Nomenclature for Interstitial Cells

Interstitial cell nomenclature has been somewhat of a battleground for many years, and it is not yet resolved. Thus it is important to provide some background on this topic and a key for readers to understand the varied terminologies found in the literature to describe these cells. Cajal described cells within villi, in the deep muscular plexus of the small intestine, and in the region of the myenteric plexus (Auerbach’s plexus in the terminology of the day) that had fusiform cell bodies and long, thin and branching, axon-like processes. The latter may have been the visual cue to Cajal suggesting that ICC were a class of primitive neuron. When Cajal’s staining techniques were repeated in more recent studies, it appears that neither glial cells nor fibroblast-like cells (including ICC) displayed this exact morphology. It has been suggested that the cells Cajal described may have been a chimeric structure consisting of glial cells or interstitial cells and contiguous nerve fibers (206). In spite of a possible disparity between the structures described by Cajal and the cells now identified as ICC, widespread usage of this term in the literature makes altering this terminology counterproductive.

Electron microscopy and histochemical techniques made it clear that ICC occupied several distinctive niches in the GI tract. Immunolabeling with c-Kit antibodies (e.g., Refs. 35, 44, 183, 377, 378) and ANO1 antibodies (35, 122, 172) clarified the distribution of ICC in specific locations and showed that ICC occupy most of the same locations in all mammals, including humans. Together with functional studies (described below), certain populations of ICC, distributed within specific planes in the tunica muscularis, can be grouped and thus linked by more specific terminology. We have called ICC within the region between the longitudinal and circular muscle layers, ICC-MY, while others refer to these cells as ICC-AP (while Auerbach’s plexus was still in common usage) or ICC-MP (since myenteric plexus has come into preferential usage). We favor the term ICC-MY because these cells are not a component of the myenteric plexus (as the terms ICC-AP or ICC-MP might imply). ICC-MY lie between the muscle layers of the myenteron and surround myenteric ganglia, but they are not
intrinsically to ganglia or the tertiary plexus. Therefore, a label implying that they are part of the myenteric plexus is morphologically incorrect. Cells within muscle bundles have been referred to as intramuscular ICC or ICC-IM. Some authors have used specific terms for ICC-IM within the circular muscle (ICC-CM) or longitudinal muscle (ICC-LM); however, no distinctive morphological or functional data exist to suggest differences between these ICC. A specialization of ICC-IM is found in the small intestine in which ICC are densely distributed and intimately associated with neurons of the deep muscular plexus, and therefore these cells are referred to as ICC-DMP. Cells at the inner aspect of the circular muscle layer in the colon and more sparsely in this location in the stomach are in contact with the submucosa and therefore called ICC-SM. Cells along the submucosal surface of the circular muscle layer are sometimes referred to as ICC-SMP, but these cells are distinct and well removed from the submucous plexus, so this designation is misleading. Cells within the septa that separate muscle bundles are referred to as ICC-SEP. TABLE 1 provides localizations of the different classes of ICC throughout the GI tract and attempts to also summarize the alternative terminologies that have been used in the literature.

When it became possible to immunolabel fibroblast-like cells with PDGFRα antibodies (174), it was recognized that this population of interstitial cells occupies most of the same anatomical niches as ICC, and therefore, similar terminology was proposed to designate these cells: 1) PDGFRα+ cells were utilized because identification by chemical coding is more specific than the vague term “fibroblast-like” cells, 2) PDGFRα+ -IM was applied to cells within muscle bundles, and 3) PDGFRα+ -MY was applied to cells in the plane of the myenteric plexus between the circular and longitudinal muscle layers (FIGURE 1, D–I; TABLE 1).

C. Role of Interstitial Cells in Pacemaking and Generation of Electrical Rhythmicity

In many, but not all, regions of the GI tract, motor patterns are intrinsically phasic in nature. Spontaneous pacemaker activity (termed, slow waves; FIGURE 4) drives and times the phasic contractile behavior. Slow waves occur in continuous rhythmic depolarization/repolarization cycles, and different regions of the bowel display distinct intrinsic slow wave frequencies (FIGURE 4, A–C). Slow waves also vary in duration from one to several seconds. Interslow wave trans-
membrane potentials (typically referred to as resting membrane potential or RMP) of GI smooth muscle cells range between −80 and −40 mV and are generally somewhat negative to the range of potentials required for substantive activation of voltage-dependent Ca$^{2+}$ channels. Thus smooth muscle cells in the SIP synctium do not generate Ca$^{2+}$ action potentials at the relatively negative potentials (RMP) between slow waves.

Activation of Ca$^{2+}$ channels provides the switch for excitation-contraction (E-C) coupling in the tunica muscularis. Slow waves are oscillations in membrane potential from base to peak of 10–60 mV. Muscles with quite negative resting membrane potentials display larger amplitude slow waves, but these events never overshoot 0 mV. When slow waves are recorded from smooth muscle cells, they rise from RMP at a peak velocity of ~1 V/s during the upstroke depolarization and then dwell in a “plateau” phase for variable durations. At completion of the plateau phase, the cells repolarize to RMP.

The primary purpose of slow waves is to depolarize smooth muscle cells sufficiently to activate Ca$^{2+}$ influx. Like vascular muscles, smooth muscle cells of the GI tract express voltage-dependent (L-type) Ca$^{2+}$ channels (86, 191, 236, 263). During slow wave depolarizations, the open probability of smooth muscle Ca$^{2+}$ channels increases and sufficient Ca$^{2+}$ entry occurs to trigger excitation-contraction coupling (281, 391). The rhythm pattern of slow waves naturally organizes the contractile activity of GI muscles into phasic contractions. In the corpus and antrum of the stomach, slow wave depolarization alone initiates peristaltic contractions, and modulation of slow wave amplitude and duration regulates the force of contractions. Depolarization for several seconds during the plateau phase of slow waves is sufficient for enough Ca$^{2+}$ entry to initiate contractions. In the pyloric sphincter, small bowel, and colon, slow waves bring membrane potentials of smooth muscle cells to a threshold for Ca$^{2+}$ action potentials, and transmembrane potential recordings from muscles of these organs typically show one or more Ca$^{2+}$ action potentials superimposed upon the peaks of slow waves (92). In these regions Ca$^{2+}$ action potentials are the smooth muscle cell response to slow wave depolarizations.

The source and mechanism of slow waves in the GI tract have been under investigation for decades. Slow waves were considered to be nonneurogenic and referred to in the literature typically as “myogenic” in origin because blocking nerve action potentials with tetrodotoxin did not abolish slow wave activity. For many years it was assumed that smooth muscle cells generated the spontaneous slow wave activity. The literature before the mid 1980s contained many studies designed to investigate the mechanism of slow waves. Some of the more detailed experiments came from the laboratories of C. Ladd Prosser and Tadao Tomita, using a variety of electrophysiological approaches including voltage clamping via sucrose gap (65, 275, 374). These studies, and much of the earlier work, were summarized in detail by Professor Tomita (373).

After development of single-cell voltage-clamp techniques, experiments on smooth muscle cells began in several labs, led by Thomas Bolton, Hiroshi Kuriyama, and the team of Josh Singer and John Walsh (25, 27, 28, 180, 222, 223, 349, 350). None of these studies, or studies performed in many other labs, recorded intrinsic electrical activity with the characteristics of slow waves from smooth muscle cells (96). Smooth muscle cells generated Ca$^{2+}$ action potentials; in some cases these occurred spontaneously, or they could be elicited by depolarization (26). In one study action potentials with plateau components were recorded from colonic muscle cells of the dog (293); however, these events were blocked by dihydropyridines, making them dissimilar to the electrical slow waves of the canine colon (411). The general conclusion from a multitude of studies was that smooth muscle cells do not have the ionic apparatus required to generate slow waves or to be the pacemaker cells in GI muscles. Thus the term myogenic is incorrect. Another cell type in GI muscles, ICC, was suggested by morphologists (98, 369) to be the pacemaker cells because these cells are electrically coupled to smooth muscle cells. However, physiological verification of this hypothesis was needed.

Dissection experiments performed on several regions of the GI tract suggested that discrete regions of tissue provide pacemaker activity for the bulk of the circular and longitudinal muscle layers. Prosser and colleagues (64, 65) noted that separation of circular and longitudinal muscles of the small intestine left circular muscles inactive, but the longitudinal muscle layer retained pacemaker activity. They suggested that slow waves originate in longitudinal muscles and are amplified and propagated by circular muscles. Later studies, in which specific strata of the circular and longitudinal muscle layers were isolated, found that slow waves were generated in the region between the circular and longitudinal muscle in the stomach and small intestine (17, 137, 185). Similar studies on colon muscles isolated two pacemaker regions in the dog: the region between the circular and longitudinal muscle generated a 17 cycle/min electrical rhythm and a thin layer of tissue at the submucosal surface of the circular muscle layer generated a 6 cycle/min rhythm (354, 355). Pacemaker activity extended into the circular muscle layer along the septa that separated the circular muscle into bundles (412). ICC networks populated all of the specialized pacemaker regions (29, 30, 412). The next tasks were to disrupt ICC networks and determine effects on slow waves and to isolate ICC to determine whether these cells were capable of pacemaker activity.

Methylene blue can be taken up by ICC in living tissues (a supravital dye), and the cells are damaged after exposure to...
high-intensity illumination (370). Damage to ICC by methylene blue exposure was associated with inhibition of slow wave activity. Methylene blue also has pharmacological effects on a variety of cells and causes, among other effects, significant depolarization of smooth muscle cells (328) and inhibition of nitric oxide synthase (416). Sufficient depolarization (e.g., with elevated external $K^+$ to the level produced by methylene blue) blocked slow waves (328). Thus the link between methylene blue labeling of ICC and the hypothesized pacemaker role of ICC was not clarified by experiments using methylene blue (370). Another supravital dye, rhodamine 123, accumulates in mitochondria, and ICC were considered to be a potential target for this dye because of their abundance of mitochondria. Rhodamine 123 is cytotoxic in some cells, so the effect of this dye on pacemaker activity was tested. Rhodamine 123 labeled ICC of the canine colon and blocked slow wave activity without the problem of substantial depolarization as observed with methylene blue (402). However, all cells have mitochondria, so cytotoxic effects in cells besides ICC may have been responsible for the effects of rhodamine 123 on pacemaker activity.

Dispersion of colonic smooth muscle tissues yielded smooth muscle cells and a few cells with an odd morphology. These cells had multiple short processes and a prominent nuclear region. Ultrastructural examination of the cells with processes showed them to have morphological features common to ICC (i.e., abundance of mitochondria, caveolae, and lack of thick filaments). Patch-clamp recordings under current-clamp conditions showed these cells generated spontaneous depolarization with characteristics similar to slow waves in colonic muscles (235). This was the first direct evidence that ICC of GI muscles have intrinsic pacemaker capability.

1. ICC as pacemakers in the small intestine

Erratic contractions of intestinal muscles from mice treated with neutralizing c-Kit antibodies and in $W/W^v$ mutants suggested defects in electrical pacemaker activity (250). Further examination of the intestinal muscles of animals treated with c-Kit antibodies showed that ICC-MY, the network of ICC in the region identified as the pacemaker area in small intestine, were largely missing (378). Loss of ICC correlated with the absence of slow wave activity in animals treated with c-Kit antibodies. Small intestinal muscles of $W/W^v$ animals also lacked most ICC-MY. Electrical recordings from these mice showed that normal slow wave activity occurred in wild-type and heterozygotes, but were absent in small intestinal muscles of $W/W^v$ mice (FIGURE 5) (167, 404). Additional studies utilizing mutants, such as $S/S^l$ mice, in which the ligand for c-Kit (steel or stem cell factor) is deficient, also displayed loss of ICC-MY and pacemaker activity (262, 403). Collectively, these studies, exploiting several paradigms of ICC loss (e.g., loss of ICC after development and developmental failure), established the role of ICC as intestinal pacemakers.

Other than the loss of ICC-MY, no other structural or developmental defects were noted in small intestines of c-Kit mutants or animals treated with c-Kit neutralizing antibod-
ies. It is also important to note that ICC in the region of the deep muscular plexus (ICC-DMP) appeared normal in W/WV and Sl/SlD mice. Intestinal motility was altered in c-Kit and stem cell factor mutants, but responses to stimulation of intrinsic enteric motor neurons were retained and smooth muscle tissues responded to neurotransmitters and generated Ca\(^{2+}\) action potentials (80, 251, 403). Complete loss of motor activity would prohibit intestinal transit, amounting to a fatal pseudo-obstruction of the bowel. However, W/WV mice live into adulthood without obvious loss of nutritional assimilation (56). A normal pattern of segmentation and peristaltic waves, observed in the small intestines of wild-type mice, was not present in W/WV mice. Intestinal muscles of these animals displayed action potentials and contractions, but these events were more random and less coordinated, leading to weakened propulsive activity (80).

Survival and apparent nutritional sufficiency in W/WV mice raises the question of the importance of ICC and slow waves. Loss of ICC-MY in W/WV mice resulted in a 10- to 17-mV depolarization in the smooth muscle syncytium (378, 403, 404). Depolarization alone was not responsible for loss of slow waves because this level of depolarization, as tested with elevated external K\(^+\), did not block slow waves (378). A second function of ICC-MY, therefore, appears to be setting membrane potential to more negative levels than would be accomplished by the intrinsicionic conductances of smooth muscle cells. Thus ICC-MY in the small intestine shift the membrane potential of the integrated SIP syncytium from a range in which smooth muscle cells generate spontaneous action potentials to more negative potentials in which the open probability of Ca\(^{2+}\) channels is reduced, and the intrinsic excitability of smooth muscle cells is therefore suppressed between slow waves. Slow waves, superimposed upon the influence of ICC-MY on membrane potential, organize the generation of muscle action potentials and phasic contractions into the phasic pattern characteristic of the small intestine. The conductances responsible for the negative membrane potentials of ICC have not yet been determined.

2. ICC as gastric pacemakers

ICC-MY are present in the gastric corpus and antrum of W/WV mice, but with application of neutralizing antibodies, lesions in gastric ICC-MY networks developed after 9–40 days in organotypic cultures (279). Slow waves were not recorded in regions of muscle where ICC-MY were lost but persisted in areas where ICC networks remained. Electrophysiological recording from guinea pig stomach also supported the hypothesis that ICC-MY are pacemakers in the stomach (82). When intracellular impalements were made, slow wave activity was recorded from circular smooth muscle cells, positively identified by dye filling during recording. More rarely, cells were impaled with much faster and larger amplitude slow wave events (termed driver potentials by these authors). The cells exhibiting driver potentials were between the muscle layers and had morphologies consistent with cells labeled with c-Kit antibodies. Simultaneous recording from ICC and nearby smooth muscle cells showed that the slow waves in ICC preceded the events in smooth muscle cells (FIGURE 6). These investigators also recorded from longitudinal muscle cells, and the slow waves in these cells were similar in waveform to events in ICC but of reduced amplitude. The slow waves recorded

**FIGURE 6.** Propagation of slow waves from ICC-MY to circular smooth muscle cells in the guinea pig stomach (trace was provided by Professor David Hirst). In the experiment illustrated, an ICC-MY was impaled (electrode 1) and a circular smooth muscle cell was impaled simultaneously. Traces above show slow wave events of large amplitude in ICC-MY and attenuated amplitude in smooth muscle cell. Note slight delay in activation of slow wave in smooth muscle cell.
from longitudinal muscle were termed follower potentials by these authors. Their results supported the hypothesis that ICC-MY generated slow waves and these events conducted to both circular and longitudinal muscle layers.

3. ICC as colonic pacemakers

ICC are reduced in the colons of W/W<sup>+</sup> mice and in genetically similar W/W<sup>−</sup> mutants in rats. However, the loss of ICC in the colon is incomplete, and therefore comparisons of activities in wild-type and mutant animals are not as clear-cut as in other regions of the GI tract. Proximal colons of wild-type rats and mice display a pattern of slow depolarization with superimposed clusters of action potentials (4). Action potential clusters were also observed in colonic muscles of W/W<sup>−</sup> rats, but these events were irregular and slower in frequency than in wild-type colons.

D. Mechanism and Modeling of Pacemaker Activity

Studies to deduce the slow wave mechanism in whole GI muscles were frustrated by the inability to record selectively from ICC. This problem was solved by the elegant work of groups led by David Hirst and Hikaru Suzuki. As mentioned above, Dickens and co-workers (82) recorded from gastric pacemaker cells and confirmed that the recordings were made from ICC-MY by filling cells with fluorescent dyes during recording. Similar recordings of pacemaker activity were made from gastric and small intestinal ICC-MY (196, 198, 199), and the mechanism of slow waves was investigated using membrane potential recordings. Experiments using I<sub>itpr1</sub><sup>−/−</sup> mice showed that loss of IP<sub>3</sub> receptor type 1 blocked gastric slow waves (364). The IP<sub>3</sub> receptor blocker 2-aminoethoxydiphenyl borate (2-APB), although not entirely specific, also blocked slow waves in ICC-MY (196). However, this effect was accompanied by significant depolarization, and sufficient depolarization alone can reduce or block slow wave generation. Treatments with a variety of pharmacological antagonists and ion channel blockers confirmed the general concept that slow waves consist of two components: the initial component (upstroke) was sensitive to Ni<sup>2+</sup> and reduced by extracellular Ca<sup>2+</sup>, and the second (plateau) was sensitive to caffeine, cyclopiazonic acid, and Cl<sup>−</sup> channel blockers (155, 196, 198, 199). Reducing extracellular Cl<sup>−</sup> also reduced the plateau potential. Multiple ionic conductances are at play in cells of the SIP syncytium. Blocking conductances or Ca<sup>2+</sup> handling mechanisms in one type of cell or altering ionic gradients might impose effects on ionic mechanisms in coupled cells. Thus definitive explanations for the pacemaker mechanism were limited until ICC could be isolated for single-cell voltage-clamp studies.

1. Studies of pacemaker activity in cultured ICC

c-Kit<sup>+</sup> cells developed in cell culture, and therefore cultured cells were used in many studies to investigate the mechanism of slow wave rhythmicity. After a few days in primary culture, single cells or networks of cells with c-Kit immunoreactivity can be identified, and these cells generated spontaneous inward currents under voltage-clamp conditions (208, 368). The inward currents reversed 10–20 mV positive to 0 mV, and the currents were not blocked by dihydropyridines. In current-clamp mode, spontaneous slow wave-like depolarization transients were observed that corresponded to the frequency of inward currents in voltage-clamp. These events displayed waveforms and mimicked some of the pharmacology of slow waves in intact muscles; however, the frequency was lower in cultured cells. Several labs have studied the ionic conductances expressed and the mechanism of spontaneous electrical rhythmicity in cultured ICC models. The inward current was generally linked to a nonselective cation conductance; however, one group suggested the inward currents were more consistent with a Ca<sup>2+</sup>-activated Cl<sup>−</sup> conductance on the basis of reversal potentials and block by SITS (372). Ion channels expressed in cultured c-Kit positive cells and concepts of electrical rhythmicity were summarized in a previous review (331), and a mathematical model was developed based on these ideas (101, 102).

Problems developed with cultured cell models of ICC. ICC make up <10% of the cells in the tunica muscularis, so enzymatic dispersion of GI muscles results in a relatively small percentage of ICC in the myriad of cells obtained. Gene or protein expression studies on these cells are likely to be no more specific than expression studies on whole muscle extracts. The relative abundance of c-Kit<sup>+</sup> cells after various periods of time in culture and the effects of growth media on retention of c-Kit expression have not been determined, so it is unknown whether the c-Kit<sup>+</sup> cell population increases or decreases relative to other cells as a function of time in culture. Some investigators have tested growing c-Kit<sup>+</sup> cells on a layer of cells expressing stem cell factor, and this promising approach enhanced the number and growth of c-Kit<sup>+</sup> cells (308). However, these studies included few evaluations of the molecular and functional phenotype of ICC. Most investigations of cultured c-Kit<sup>+</sup> cells have been performed without the benefit of a reporter molecule expressed in ICC for the purpose of unequivocal identification. Initial labeling with antibodies to confirm the presence of c-Kit immunopositive cells (208, 368) is not performed on all cells sampled for electrical recording. Cells were selected for recording on the basis of gross morphology. Thus it is possible that the cells from which molecular evaluations or physiological recordings were made were confused with other cell types in mixed cell populations from which the cultures were grown. Some investigators have performed purification steps before culturing, such as immunoselection or FACS (276); however, tests to certify cell purity have been rather limited.
As networks of c-Kit+ cells develop in culture, they experience changes in phenotype within a few days. However, few tests verifying the fidelity of the native phenotype have been performed on cultured c-Kit+ cells. A genomic study of freshly dispersed ICC from the murine small intestine produced a catalog of highly expressed genes that could provide a standard for validation of the ICC phenotype (52). Our own experience with cultured ICC was that the conductances responsible for slow waves in situ were lost within a few days in primary culture (436). Therefore, we have concluded that the mechanisms of rhythmicity reviewed previously (331) may largely be artifacts of cell culture. This experience should raise caution about conclusions based on studies of cultured c-Kit+ cells that are not accompanied by careful verification that the mechanisms and pathways observed are equivalent in the native phenotype. For this reason, little space in this review is devoted to the many ion channels, agonist responses, and slow wave mechanisms that have been reported from studies of cultured c-Kit+ cells.

2. Studies of pacemaker activity in freshly isolated ICC

A main concern about the activity recorded from cultured ICC-like cells was that they lacked the ability to be paced by application of current pulses. This was a fundamental and important difference between the behavior of cultured cells and whole muscles, which can be paced (296). Due to skepticism about the behavior of cultured cells, Akinori Noma and colleagues (125) sought to study ICC from the myenteric region (pacemaker region) of the mouse small intestine. These authors developed a unique dispersion technique that did not disrupt the anatomical orientation of the cells from myenteric plexus region but liberated cells that could be voltage clamped. ICC-MY, verified by immunolabelling of unfixed tissues, had an average whole cell capacitance of 25 pF. ICC had resting potentials of −72 mV and generated spontaneous transient depolarizations under current clamp and large inward currents under voltage clamp. The inward currents were termed “autonomous currents,” because once these currents were initiated by depolarization, they displayed an autonomous time course of −500 ms. Autonomous currents were attributed to a non-selective cation conductance. Autonomous currents displayed what appeared to be inactivation properties, but inactivation was characteristically different from the Marcovian behavior of voltage-dependent ion channels. It was concluded that activation and deactivation of autonomous currents depended on intracellular mechanisms that responded to depolarization and regulated channel openings. It was also shown that replacing the extracellular solution with one that was nominally free of Ca2+ caused gradual reduction in the amplitude of autonomous currents, suggesting that Ca2+ stores were an important component in activation of autonomous currents. Demonstration of autonomous currents in freshly isolated ICC was an important step in understanding the cellular mechanism of pacemaker activity and a potent indication of the loss of function occurring via remodeling of ICC in cell culture.

Production of a reporter strain of mice in which ICC were labeled constitutively by expression of a green fluorescent protein (Kit+/copGFP) made it possible to identify ICC in mixed cell dispersions of the tunica muscularis and perform experiments soon after enzymatic dispersion (311, 436). Tmem16a (official name now is Ano1) had previously been identified as one of the most highly expressed genes in ICC by a gene array screen of the ICC transcriptome (52), but the function of the protein encoded by Tmem16a was unknown at the time. In 2008 the gene product of Tmem16a was found to be a Ca2+-activated Cl− channel (Anoctamin 1 or ANO1) (48, 336, 426). Immunohistochemical studies showed that ANO1 was highly expressed in ICC of several species, including humans (122, 163, 172).

ICC freshly isolated from Kit+/copGFP mice display a large-amplitude inward current that was activated by depolarization and followed a time course similar to the current previously identified as “autonomous current” (125). However, the reversal potential of the current in freshly isolated ICC indicated that a Cl− conductance was responsible for the large-amplitude inward currents activated by depolarization (436). The Cl− conductance displayed unusual activation properties: a very sharp increase in current was noted with depolarizations in the range of −70 and −50 mV and a voltage-dependent delay in activation was observed at near-threshold potentials. At potentials positive to the activation threshold, the current developed to maximum amplitude and was linearly dependent on the Cl− gradient. The voltage dependence of activation was not fit by a Boltzmann function, and therefore, the underlying conductance was not likely to be voltage dependent. Activation of the conductance was blocked by Ni2+, replacement of extracellular Ca2+ with Ba2+, or exclusion of Ca2+ from the extracellular solution. These and other experiments suggested that activation of the inward current was due to an initial transient influx of Ca2+, and the delay in activation with near-threshold depolarizations may have been due to minimal Ca2+ influx at more negative steps. ICC also expressed a single-channel, Ca2+-activated Cl− conductance of 7.8 pS, which is similar to the conductance of ANO1 channels expressed in HEK cells (426). ICC currents were blocked by niflumic acid with an IC50 of 4.8 μM. Under current clamp, ICC discharged spontaneous depolarizations that mimicked the properties of slow waves in intact muscles. The spontaneous depolarization events were also blocked by niflumic acid. As described in the next paragraph, the Ca2+-activated Cl− conductance appears to be responsible for slow waves in GI muscles, so the current activated in single ICC was referred to as the “slow wave current.”
Experiments on intact muscles suggested that a component of slow waves was due to a Ca\(^{2+}\)-activated Cl\(^-\) conductance (e.g., the plateau phase of slow waves was blocked by treating tissues with membrane permeable Ca\(^{2+}\) buffers, niflumic acid, and by removal of extracellular Cl\(^-\)) (153, 198). Cl\(^-\) channel blockers were also found to reduce the frequency and block slow waves in murine, monkey, and human small intestine and stomach (172). However, it should be noted that the concentration sensitivity for these effects varied between organs and species with gastric muscles of monkeys being the most sensitive to niflumic acid. ICC express several splice variants of Ano1 (172), and it is possible that the variable sensitivity to Cl\(^-\) blockers and/or the differences in the waveforms of slow waves might be due to variations in the complement of Ano1 isoforms expressed in different regions of the GI tract. The role of ANO1 channels in slow waves was further tested using \(Tmem16^{-/-}\) (\(Tmem16^a\)tm1Bdh/tm1Bdh) mice (313). Previous studies had shown that ~80% of mice with congenital deactivation of ANO1 channels died within a week after birth. Experiments were performed, therefore, on newborn animals and after slow waves developed further in organotypic cultures (406). All mice of several litters were assayed for electrical slow waves by intracellular recording and then genotyped later for \(Tmem16^a\)tm1Bdh alleles. Newborn \(Tmem16a^{-/-}\) mice displayed no slow wave activity while normal slow wave activity was recorded from mice with one or both wild-type alleles. After 6 days in organotypic culture, slow waves developed and increased in amplitude in mice with wild-type alleles but were absent in muscles of \(Tmem16a^{-/-}\) mice. Mice of some litters were allowed to develop for up to 23 days after birth. No slow waves were recorded from gastric and small intestinal muscles from \(Tmem16a^{-/-}\) mice, but normal slow waves were recorded from siblings with wild-type alleles. These data strongly support the hypothesis that Cl\(^-\) channels, encoded by Ano1 (\(Tmem16a\)), are responsible for slow waves in GI muscles and justify the term slow wave current to describe the large-amplitude inward currents initiated spontaneously and by depolarization of single ICC.

3. The “clock” driving the pacemaker mechanism in ICC

Insight into the clock mechanism driving slow waves was provided by studies on gastric muscles from the guinea pig (155). The intervals between slow waves were tabulated and found to vary around a fairly tight mean value, and the interval was not dictated by neural inputs to the muscle. The interval between slow waves was tightly linked to the duration of slow waves, and the probability of initiating the next slow wave increased from low values immediately after repolarization of the past slow wave until the next event. The increase in excitability was linked to the discharge of small-amplitude spontaneous transient depolarizations (STDs, or “unitary potentials,” as termed by the authors). The increase in probability of STDs was associated with activation of a full-amplitude slow wave. STDs are commonly recorded from cells within intact networks of ICC in situ (155, 196–199, 383) and can be conducted to and recorded from smooth muscle cells in small strips of muscle (21, 45, 153, 197). STD activity is lost from smooth muscle recordings when ICC are absent (45). Single ICC also generate STDs, and these events are due to transient activation of Ca\(^{2+}\)-activated Cl\(^-\) currents (436, 437). Stochastic generation of STDs in ICC within networks of pacemaker cells (155) leads to activation of the voltage-dependent process linked to activation of slow wave currents (436). There is still speculation about the nature of the voltage-dependent process. Our group has favored voltage-dependent Ca\(^{2+}\) entry, mainly via T-type Ca\(^{2+}\) channels, as the voltage-dependent mechanism (194, 433) because reduced extracellular Ca\(^{2+}\), Ni\(^{2+}\), and mibebradil can block slow wave generation and slow or block the propagation slow waves in intact muscles and generation of slow wave currents in single ICC (20, 405, 411, 413, 436). The \(\alpha1\)H isoform (\(Cacna1b\)) has been suggested as the T-channels responsible for slow wave propagation (120). Others have suggested a mechanism involving voltage-dependent activation of IP\(_3\) formation or voltage-dependent sensitization of IP\(_3\) receptors (153, 273, 383). More experiments will be needed to resolve this controversy.

It is important to note that every type of ICC from which electrical recordings have been made displays STDs; however, only some ICC are capable of generating slow waves (196–199, 436, 437). This suggests that the basic pacemaker mechanism (i.e., STDs) is an intrinsic feature of ICC; however, some ICC lack the voltage-dependent mechanism(s) required for STDs to summate into slow wave currents or to propagate slow waves actively (see section below). The simplest explanation for the inability of some classes of ICC to generate slow waves is that these cells lack the voltage-dependent mechanism(s) responsible for initiation of slow wave currents (see FIGURE 7).

Ca\(^{2+}\) handling mechanisms that control activation of ANO1 channels are not well understood at present. It seems clear that release of Ca\(^{2+}\) from Ca\(^{2+}\) stores via IP\(_3\) receptors is required for generation of slow waves (364), but sources of Ca\(^{2+}\) for filling of stores, initiation, or amplification of Ca\(^{2+}\) transients by Ca\(^{2+}\) entry or ryanodine receptors, and the role of Na\(^+\)/Ca\(^{2+}\) exchange are controversial. The Ca\(^{2+}\) sensor that is either intrinsic to ANO1 or provided by an accessory protein has also not been identified. Finally, the role of excluded volumes that might occur because of close apposition between stores and the plasma membrane are not understood. At present, our best concept of the slow wave mechanism is illustrated in FIGURE 7. In spite of many uncertain elements, there have been numerous recent attempts at modeling pacemaker behavior in ICC (42, 101, 102, 384, 428). Simulations of pacemaker activity of ICC are improving, and the models and refinements provided by...
1. Generation of STICs (All ICC)

2. Generation of slow wave currents (Pacemaker ICC)

3. Chronotropic enhancement of pacemaker current (ICC-IM or ICC-MY)

**FIGURE 7.** A model of electrical rhythmicity based on studies of freshly isolated ICC of the murine small intestine (433, 436, 437). Figures show portion of plasma membrane in close association with Ca$^{2+}$ release mechanisms of the endoplasmic reticulum (ER). 1: The basic event of electrical rhythmicity is spontaneous transient inward currents (STICs) elicited by localized release of Ca$^{2+}$ from stores. IP$_3$-gated channels (IP$_3$R) are the main source of Ca$^{2+}$ driving activation of Ca$^{2+}$-activated Cl$^{-}$ channels (ANO1) in the plasma membrane to generate STICs, but ryanodine receptor (RyR) may also contribute. Ca$^{2+}$ is recovered into stores via active Ca$^{2+}$ pumping into the ER. STICs generate spontaneous transient depolarizations (STDs), the voltage response to the inward currents. 2: The second phase of rhythmicity is activation of T-type voltage-dependent Ca$^{2+}$ channels (VDCC) in response to the STDs caused by STICs. Ca$^{2+}$ entry synchronizes Ca$^{2+}$ release in other regions of membrane, leading to synchronized opening of ANO1 channels throughout the cell. This generates slow wave currents. Depolarization also activates VDCC in adjacent electrically coupled cells, leading to cell-to-cell active propagation of slow waves in ICC networks. 3: In the 3rd phase of rhythmicity, binding of chronotropic agonists (such as ACh in this example) to G protein-coupled receptors (type 3 muscarinic receptor, M3, in this example) leads to IP$_3$ formation and increased frequency and amplitude of STICs. More frequent STICs and greater amplitude depolarizations increase the likelihood of activation of whole cell slow wave currents. The steps in this mechanism are deduced from studies on ICC of the murine small intestine. Pharmacological data suggest that the generalized mechanism proposed applies to other GI muscles (e.g., dependence upon Ca$^{2+}$-activated Cl$^{-}$ currents to generate STICs and slow wave currents); however, the mechanism for regulation of frequency is poorly understood and appears to vary in ICC from different regions of the GI tract and the mechanism for voltage-dependent Ca$^{2+}$ entry and/or release, necessary for slow wave propagation, appears to vary. For example, slow waves recorded from different regions of the murine GI tract and in other species display a range of sensitivities to extracellular Ca$^{2+}$, Ni$^{2+}$, and dihydropyridines. The ionic and molecular explanations for the diversity in the slow wave mechanism and for regulation of frequency are not fully understood.
further experimentation will be very useful in planning future experiments and in predicting the actions of therapeutic agents on electrical rhythmicity in the gut.

E. Role of ICC in Propagation of Slow Waves

1. Extracellular electrical recording from visceral smooth muscles

A great deal of the information about GI pacemaker activity used by gastroenterologists to make diagnoses, biomedical scientists in testing hypotheses and drugs, and instructors for teaching GI physiology has been deduced from studies using electrical recordings made with extracellular metal electrodes laid against or fastened to the serosal or mucosal surfaces of GI organs or applied to the abdomen (i.e., electrogastrograms). Studies of this sort have been performed for almost 100 years; however, a recent report raises questions about the validity of results obtained with this method (18). Activity was recorded from murine gastric sheets in an attempt to relate electrical activity to the aberrant motility patterns in mutant mice with dysmotility phenotypes. These studies utilized monopolar or bipolar recording via silver electrodes arranged into arrays, as used commonly in many recent studies (e.g., Refs. 87, 227, 274, 390). Biopotentials detected by these electrodes were monitored by standard techniques, and it was noted that the frequency of gastric slow waves was significantly lower than the frequencies observed when intracellular electrical recording were made from intact gastric sheets (103, 104). When contractions were blocked by dihydropyridines or wortmannin (both drugs chosen to block contractions downstream of the mechanism that generates slow waves), extracellular biopotentials were abolished. Impalement of cells in muscles, however, showed that the drugs had no effect on slow wave amplitude or frequency. By combining imaging of muscle movements with extracellular recording, it became apparent that very small muscular movements (<50 μm) were capable of generating voltage transients several tens of microvolts in amplitude (i.e., equivalent to the events typically considered to be slow waves). Thus extracellular electrical recording from visceral smooth muscles may be largely artifacts of contractile movements. In exploring the lengthy literature in which extracellular recording has been used to monitor the electrical activity of the GI tract, it is apparent that controls for movement artifacts have been neglected. Thus it is reasonable to be skeptical about the results of classic studies that have based major conclusions upon this technique. Future investigations using extracellular recordings will need to demonstrate the validity of these recordings by control experiments in which recording fidelity is maintained after rigorous stabilization of movement. A recent study claiming maintenance of extracellular recording fidelity after infusion of a dihydropyridine into pig intestine in vivo relied only upon visual inspection to ensure that movement was blocked (10). This study, while clearly seeking to prove the fidelity of extracellular recording, falls well short of a suitable control study for extracellular recording, because: 1) dihydropyridines don’t block all contractile movements in many species, 2) no concentration/effect data for dihydropyridines were provided, 3) movements of the viscera due to respiration and pulsatile blood flow could not possibly have been absent in a living animal, and 4) more rigorous monitoring of movement than visual inspection would be needed to detect the small movements capable of eliciting electrical artifacts.

Difficulties in recording authentic electrical activity in GI muscles with extracellular electrodes are likely due to the structure of ICC networks, the fact that ICC capable of regenerative propagation are buried within muscle layers, and the magnitude and kinetics of transmembrane currents responsible for slow waves. In contrast to the heart, in which cardiac myocytes actively regenerate action potentials along a coherent wave front, the gut has a thin and relatively sparse network of ICC generating the transmembrane currents responsible for slow waves. The velocity of the upstroke phase of slow waves is ~100-fold less than the upstroke velocity of cardiac action potentials, indicating much lower current densities during slow wave depolarizations. Field potentials resulting from slow waves are far weaker than the field potentials generated by cardiac and skeletal muscles or neurons and may be lost in the noise of recording.

2. Propagation of slow waves in intact muscle strips

Motility patterns in the gut depend on coordination of millions of smooth muscle cells. For example, in gastric motility, slow waves originate in the proximal corpus and peristaltic contractions spread over many square centimeters of the stomach. Timing of contractions is accomplished by active slow wave propagation, and several studies have characterized propagation using intracellular recordings to determine propagation velocities of slow waves initiated from known sites of pacing. These studies have typically used muscle strips or sheets from larger animals to obtain cable lengths sufficient to resolve temporal separation of slow waves recorded from two points. In the canine antrum, for example, slow wave propagation velocity was measured at 20–60 mm/s in the circumferential direction (long axis of circular muscle fibers) and ~10 mm/s in the longitudinal direction (long axis of longitudinal muscle fibers) (17, 20). The precise explanation for the anisotropy in propagation velocities is unknown. Slow waves display refractory properties, as do excitable events other in excitable cells and cell networks. In the canine antrum, the period between slow waves required for complete restoration of slow wave upstroke velocity, amplitude, and duration is at least 10 s (296), demonstrating the long latencies required for resetting Ca\(^{2+}\) entry/release mechanisms and ion channels re-
quired for slow wave generation and propagation. In fact, ion channel recovery from inactivation is categorically much quicker than the long refractory period of slow waves, suggesting that other mechanisms, such as reloading of intracellular Ca²⁺ stores, is likely to be the rate-limiting step in slow wave frequency and responsible for long periods of refractoriness.

ICC have a critical role in slow wave propagation because these cells provide the pathway for active propagation in GI muscles. Smooth muscle cells lack the ionic apparatus necessary to regenerate slow waves. Thus a coupled network of ICC is required for coherent propagation of slow waves and coordination of contractions (FIGURE 8). This point was demonstrated by dissection experiments in which bits of tissue containing pacemaker cells were removed from muscles of the canine colon. In intact muscles, slow waves of uniform amplitude were recorded from cells along the submucosal surface of the circular muscle layer, and active propagation of slow waves occurred in this region. Slow wave activity was lost if a thin band of tissue along the submucosal surface (containing pacemaker ICC-SM) was removed, and slow waves decayed within a few millimeters from a region with the pacemaker cell network intact (332). A similar phenomenon was observed in gastric muscles treated with neutralizing c-Kit antibodies, slow waves were generated in areas where clusters of ICC were retained, but these events did not propagate to regions devoid of ICC (279). Furthermore, after loss of ICC, gastric muscles could not be paced by application of current pulses.

Recordings of slow waves in ICC and from nearby smooth muscle cells demonstrate how slow waves conduct via electrical coupling into smooth muscle cells. Large-amplitude slow waves were recorded in ICC, and much attenuated slow waves were recorded from nearby smooth muscle cells.

**FIGURE 8.** Active propagation of slow waves. 1: The initiating step occurs by localized Ca²⁺ transients (elevated Ca²⁺ depicted by green color in all cells; arrow), initiating STICs in an ICC. 2: Depolarization caused by STICs activates Ca²⁺ entry and Ca²⁺-induced Ca²⁺ release raising Ca²⁺ throughout the ICC and activating whole cell slow wave current. 3: Depolarization causes active propagation of slow waves through the ICC network (horizontal black arrow shows direction of propagation), and slow wave currents are activated cell to cell, as the wavefront spreads. 4: As slow waves propagate through the ICC network, they conduct passively into electrically coupled smooth muscle cells (SMCs). Slow waves depolarize SMCs and activate L-type Ca²⁺ channels. Ca²⁺ entry (green) triggers SMC contraction. Spread of slow waves in the ICC network leads spread of contractions necessary for segmental and peristaltic contractions.
Hirst and colleagues (68) described the electrical coupling between ICC and smooth muscle cells as weak and showed that conduction of slow waves is similar to conduction of electrotonic potentials between these cells. Taken together, these studies suggest that slow waves, generated by the ICC, conduct passively into the smooth muscle syncytium and are not actively regenerated by smooth muscle cells. Thus active (regenerative) propagation of slow waves by ICC-MY is another central function of these cells. Unlike the heart that is paced from discrete clusters of pacemaker cells and action potentials propagate via the regenerative mechanisms in cardiac myocytes, the gut requires a continuum of ICC for regenerative (active) propagation of slow waves. This feature has important consequences in pathophysiological circumstances where loss of some ICC might uncouple regions of ICC networks or reduce the safety factor for propagation, thus preventing normal coherent spread of slow waves and causing loss of contractile coordination.

A comment should be included about the form of electrical coupling between ICC-MY and smooth muscle cells because some authors have claimed that gap junctions between these cells cannot be found by electron microscopy and have proposed alternative methods of electrical coupling, including structures called peg-and-socket junctions (371) or electrical field coupling (360). Whether some form of ephaptic communication could be effective in conducting slow waves from ICC-MY to smooth muscle cells seems unlikely and has not been demonstrated. Gap junctions between these cells may be rare and small in size, and these structures have been observed between ICC-MY and smooth muscle cells in some species (331). Direct electrical measurements have shown that electrical coupling between ICC-MY and smooth muscle cells is weak (68), and this may result from few and small gap junctions.

ICC-IM in corpus and antral muscles also appear to have regenerative capabilities that can sustain circumferential propagation of slow waves. Hirst and colleagues (152, 154) noted that ICC-MY formed a dense network along the greater curvature, but this population of ICC was greatly diminished or missing near the lesser curvature in rodent stomachs. In spite of the decrease in ICC-MY, slow waves propagated from the greater curvature to the lesser curvature with little decrease in amplitude (152). These authors also found that ICC-IM in the corpus were capable of a relatively fast rate of spontaneous slow wave generation and suggested that these cells are the source of the higher frequency slow waves in the corpus. They viewed activation of the gastric musculature in the following manner: 1) ICC-IM generate corporal slow waves, and these events determine the dominant frequency in the stomach; 2) slow waves from ICC-IM activate slow waves in the ICC-MY network, and these events spread down the stomach; 3) slow waves conduct into bundles of smooth muscle cells, elicit depolarization, and initiate contractions; and 4) depolarization also initiates active responses in ICC-IM (referred to as regenerative potentials) which spread rapidly along the long axis of these cells in circumferential bands (81). In order for this mechanism to work, ICC-IM in the corpus and antrum must have a voltage-dependent mechanism that is primed to provide cell-to-cell regeneration of slow waves. Cellular studies of ICC-IM have been limited, but a voltage-dependent mechanism intrinsic to ICC-IM has not yet been identified. Such a mechanism could be present in ICC-IM or in the septal ICC (ICC-SEP) that run between bundles of smooth muscle cells because small bundles of gastric muscle, that likely contain both ICC-IM and ICC-SEP, are capable of regenerative propagation (383).

There may be significant differences between the ICC-IM in different parts of the stomach. In laboratory animals ICC in fundus muscle bundles do not display voltage-dependent regenerative mechanisms (21), whereas the ICC within bundles in the distal stomach can generate active responses to depolarization or upon break from hyperpolarization (153, 383). There are clearly many things to learn about the interactions between ICC and the specific ionic properties of different classes ofICC before slow wave propagation is fully understood or can be modeled accurately.

### 3. Ca^{2+} imaging to study mechanism and propagation of pacemaker activity

Intracellular Ca^{2+} transients are responsible for the spontaneous transient inward currents (STICs) that generate STDs in ICC. Depolarization from STDs triggers global Ca^{2+} transients that activate whole cell slow wave currents. Imaging of Ca^{2+} transients with Ca^{2+} sensitive dyes has been used to characterize propagation of pacemaker activity in networks of ICC in situ (146, 283). ICC take up Ca^{2+} indicators, such as fluo 4, making it possible to resolve cyclical Ca^{2+} transients in ICC that correspond to slow waves, and simultaneous recording of Ca^{2+} transients and slow waves showed 1:1 correlation between these events. Ca^{2+} transients provide a window and cell-specific detail on pacemaker activity that is not possible with electrical recording. Studies using ICC-specific expression of molecular probes, such as GCaMP, will be a powerful new technique for studying slow wave propagation in intact GI muscles and organs.

Ca^{2+} imaging studies on guinea pig and mouse tissues in vitro reinforced the concept that slow wave generation is stochastic in nature (155), because events within a network of ICC in sheets of muscle are generated from variable points and propagation within a network occurs via often changing pathways (146, 283). Activation of a network of ICC is directed from the first cell to reach threshold, but this position of leadership changes from cycle to cycle. Creation of spatiotemporal maps from video images allows visualization of direction and velocity of activity fronts. Ca^{2+}...
imaging also reinforced the pacemaker role for ICC-MY because Ca\(^{2+}\) transients in closely spaced ICC-MY and smooth muscle cells occur sequentially (Figure 9), as would be expected if slow waves, generated in ICC-MY, initiated depolarization and activation of voltage-dependent Ca\(^{2+}\) channels in neighboring smooth muscle cells (146, 147, 283). Average propagation velocities of wave fronts in ICC networks in the mouse were between 2–6 mm/s, and therefore consistent with the velocities of slow wave propagation in murine muscles. The propagation velocity of Ca\(^{2+}\) transients associated with slow waves greatly exceeds the rates of Ca\(^{2+}\) waves that result only from diffusion (10–50 μm/s) (367), supporting the idea that slow wave propagation depends on a voltage-dependent mechanism.

The mechanism of propagation was investigated by imaging of Ca\(^{2+}\) dynamics in ICC networks, and these studies showed that electrical coupling between cells was fundamental to the organization of propagating Ca\(^{2+}\) transients (283). Treatment of ICC-MY networks with 18β-glycyrrhetinic acid, a gap junction uncoupler, did not block Ca\(^{2+}\) transients in individual cells, but blocked the spread of transients through the network. After 18β-glycyrrhetinic acid, individual cells paced at their intrinsic frequencies, which varied from cell to cell, and failed to be entrained by a dominant pacemaker frequency in the network. By tabulating the time course of Ca\(^{2+}\) transients in multiple cells within a network, it was possible to characterize the cellular sequence of activation and the time course of cellular transients event to event. This analysis showed that the activation sequence (i.e., spread of slow waves through a network) varies even when the direction of propagation remains constant. The time lag in activation between adjacent cells also varied from cycle to cycle, likely reflecting the dynamics of refractoriness and excitability of individual cells. These properties are consistent with the cablelike properties of a complex two- or three-dimensional network of excitable cells.

Imaging studies also support the hypothesis that the voltage-dependent mechanism for propagation is Ca\(^{2+}\) entry through voltage-dependent, possibly T-type Ca\(^{2+}\) channels (283). Nicardipine did not affect Ca\(^{2+}\) transients in ICC-MY, but greatly reduced or blocked Ca\(^{2+}\) transients in smooth muscle cells. Ni\(^{2+}\) (100 μM) severely disrupted Ca\(^{2+}\) transients, and mibefradil blocked Ca\(^{2+}\) transients propagating through ICC networks. Drugs, such as cyclopiazonic acid (10 μM) and 2-APB (50 μM), which affect intracellular Ca\(^{2+}\) handling mechanisms, reduced the frequency of ICC-MY Ca\(^{2+}\) transients, and ryanodine (10 μM) did not affect frequency but reduced the amplitude of these events. These data are consistent with the idea that Ca\(^{2+}\) entry entrains the discharge of localized Ca\(^{2+}\) release events, generates whole cell Ca\(^{2+}\) transients, and facilitates propagation of slow wave currents in ICC networks.

Imaging has also been used to study pacemaker activity and propagation in larger animals and human muscles, and these studies have allowed generalizations to be made about the role of ICC in pacemaker activity and the mechanism for pacemaking. For example, studies on muscles of the human jejunum showed Ca\(^{2+}\) transients in ICC-MY with characteristics similar to the events in murine muscles (241). The Ca\(^{2+}\) transients in human ICC-MY were biphasic in nature, and the initial component was sensitive to Ni\(^{2+}\) and mibefradil. Ca\(^{2+}\) transients in human jejunal ICC-MY were not affected by dihydropyridines, but were reduced or blocked by cyclopiazonic acid and 2-APB. Because slow waves spread passively (i.e., with decrement) into smooth muscle bundles, electrical events cannot penetrate very far into the depth of thicker muscles of larger animals and humans (412). However, ICC lying within the septa (ICC-SEP) between muscle bundles in these tissues

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**FIGURE 9.** Spread of Ca\(^{2+}\) transients in the ICC network of murine small intestine. ICC were loaded with fluo 4. Sequence of video images shows development of Ca\(^{2+}\) transient in ICC-MY and spread of wave front from upper left quadrant to whole image. Streaks of fluorescence are longitudinal smooth muscle cells (LM) that are above the ICC-MY network. Note that ICC fire Ca\(^{2+}\) transients prior to events in LM. Ca\(^{2+}\) transients in regions of interest in ICC-MY and LM cells are shown as traces as a function of time in the bottom right of figure. Spatiotemporal maps were constructed from repetitive Ca\(^{2+}\) transients sweeping through the ICC-MY network. [Redrawn from Hennig et al. (147). Copyright 2010 Blackwell Publishing Ltd.]
were found to activate sequentially as a function of distance from ICC-MY in the human small intestine (242). Thus propagation of slow waves through ICC-SEP drives depolarization of smooth muscle cells deep within smooth muscle bundles.

There are two pacemaker regions in the canine colon, each producing different frequencies of pacemaker events (354). Interesting differences were observed in the activation and propagation of Ca\(^{2+}\) transients in ICC in the submucosal pacemaker area (ICC-SM), that produce slow waves, and ICC-MY, that produce faster oscillations, called myenteric potential oscillations. Rhythmic Ca\(^{2+}\) transients (5–8/min) spread without decrement through the ICC-SM network, but Ca\(^{2+}\) transients in ICC-MY (16/min) were typically unsynchronized in adjacent cells. Excitatory neural inputs synchronized the Ca\(^{2+}\) transients in ICC-MY. These findings demonstrate the diversity of behaviors and pacemaker frequencies found in ICC from different parts of the GI tract. Why ICC with different pacemaker frequencies, voltage dependencies, and synchronization between cells develop in different regions of the GI tract and what factors are responsible for pacemaker diversity are unknown at the present time.

4. Relating Ca\(^{2+}\) transients in ICC to motor patterns of GI organs

Comparing the activation and propagation of Ca\(^{2+}\) transients in wild-type and W/W\(^{V}\) mutants provided a means of determining the role of ICC-MY in regulating motor patterns in the small intestine (FIGURE 9) (147). In the mouse Ca\(^{2+}\) transients in ICC-MY precede transients in adjacent longitudinal muscle fibers by 66–127 ms. The frequency of Ca\(^{2+}\) transients in muscle fibers (30 cpm), variability in the intervals between transients (−7%), and propagation velocities (−4 mm/s) in the longitudinal muscle layers of wild-type mice were similar in flat sheets of tunica muscularis, isolated intestinal segments, or exteriorized loops of intestine. In exteriorized loops Ca\(^{2+}\) transients were observed around the circumference of the intestine and were constrained within a band ~1.5 mm in length. Ca\(^{2+}\) transients in longitudinal muscle cells preceded longitudinal shortening and circular contraction by ~1 s. Contractions in wild-type mice were stable, most prominent in the longitudinal axis, and originated at the oral ends of preparations. Forceful contractions were also observed in muscles of W/W\(^{V}\) mice; however, contractions were abrupt in onset and uncoordinated compared with the contractile patterns of wild-type mice. The velocity of propagation of Ca\(^{2+}\) transients in longitudinal muscles was about six times faster than propagation in wild-type small intestine, and origination sites of Ca\(^{2+}\) transients were highly unstable. Ca\(^{2+}\) transients propagated over smaller areas, and frequent collisions of Ca\(^{2+}\) transients were apparent.

ICC-MY facilitate coherent propagation of electrical activity which provides organization for muscle depolarization, Ca\(^{2+}\) transients, and contractions (147). When ICC are reduced in number, smooth muscle cells escape from the discipline of slow waves and revert to intrinsic excitability mechanisms. After loss of slow waves, smooth muscle cells generate spontaneous action potentials, and there is little segment-wide regulation of the timing of action potentials. Thus the motor behavior in the absence of ICC-MY differs considerably from normal motility patterns. Contractions are less circumferential, directional organization of contractions decreases, and action potentials and contractions generated from random sites often collide.

Slow waves can propagate for long distances through ICC networks, providing organization of contractile activity at the organ level. The time constants of the SIP syncytium do not favor propagation of action potentials, and therefore, regenerative propagation of action potentials tends to be limited to a small cluster of cells (2). Smaller regions of muscle, synchronized only by action potentials, produce less organized and weakened contractions observed in small intestines of W/W\(^{V}\) mice (80). Thus, while muscles and organs lacking ICC-MY still generate spontaneous electrical activity and contractions, the loss of the rhythmic depolarization-repolarization cycle provided by clock-like slow waves leads to abnormal and less efficient intestinal motility.

F. Role of Interstitial Cells in Neurotransmission

An early idea proposed by Cajal (47) was that interstitial cells are primitive neurons providing connectivity between autonomic nerve fibers and effector tissues. We now know that ICC are not neurons and do not develop from precursors from the neural crest as do the neurons and glia of the enteric nervous system (238, 379, 430). However, the idea that ICC mediate inputs from enteric motor neurons has developed dramatically in recent years. This idea has been a controversial topic in ICC research because several investigators have reported that contractile responses to nerve stimulation are retained in animals lacking some or most of the ICC thought to mediate neural inputs (ICC-IM). The debate about the role of ICC in neurotransmission was discussed in recent reviews (126, 329) and is summarized in this review. Resolving this controversy is important because regulating motor input to visceral smooth muscle organs may provide a means for therapeutic regulation of motor activity. Understanding the postjunctional mechanisms for transduction of neural inputs and how these signals are linked to excitation-contraction coupling are fundamental to understanding the reasons for many motor defects in the GI tract.
Some types of ICC (and PDGFRα+ cells, as discussed below) are distributed in a manner that would facilitate innervation by motor neurons (FIGURE 1, G–I). The cells termed ICC-IM (or ICC-DMP in small intestine) and PDGFRα+ cells alone lie along the projections of excitatory and inhibitory motor neurons within bundles of muscle fibers in most regions of the gut. These interstitial cells form gap junctions with smooth muscle cells. Close associations with varicosities of motor neurons and electrical connectivity with smooth muscle cells are suggestive, but not proof, of a role for these cells in neurotransmission or neuromodulation. Morphologists noted very close contacts between ICC and varicosities of enteric motor neurons with electron microscopy, and Imai and Hama (179), for example, suggested that “...the interstitial cell may play a role in transmitting stimuli received from the axon to surrounding smooth muscle cells by an electrotonic response.” This comment, and the original idea of Cajal, stimulated further investigation of ICC in neurotransmission. A morphometric study of the lower esophageal sphincter performed by Edwin Daniel and Virginia Posey-Daniel revealed an abundance of extremely close contacts (<20 nm) between varicosities and ICC and far fewer of these contacts between varicosities and smooth muscle cells (73). These authors suggested that if “intercalation” of ICC between motor nerve terminals and smooth muscle cells was prevalent, then “...attempts to define the characteristics of nonadrenergic, noncholinergic junction potentials in smooth muscle and compare them with effects of putative mediators may be misleading. If the smooth muscle junction potential is produced after electrotonic transmission from interstitial cells and its characteristics are determined by events in the interstitial cells, then there is no reason to expect coincidence of effects of nerve-released and exogenously added mediators. The first may act directly on interstitial cells and the second on smooth muscle cells as well as on interstitial cells, with the action on smooth muscle cells likely to be predominant.” This comment turned out to be an important signpost in the course of study of enteric motor neurotransmission.

1. Loss of excitatory and inhibitory neurotransmission in muscles of W/WV mice

In most regions of the GI tract, ICC-IM are closely associated (<20 nm gaps) with varicosities of motor neurons and form gap junctions with adjacent smooth muscle cells. ICC-IM are greatly reduced in numbers in gastric muscles of W/WV mice (45). Electrical field stimulation of wild-type muscles evokes excitatory and inhibitory junction potentials in the fundus, and both nitricergic and cholinergic junction potentials were reduced significantly in muscles of W/WV mice with reduced ICC-IM (45, 365, 400). Loss of these responses was not due to failure of motor neurons to develop and innervate muscle bundles or to release neurotransmitters, and responses were not lost because smooth muscle cells lost sensitivity to ACh and nitric oxide (NO). Contractile responses to exogenous transmitters were similar in wild-type and W/WV muscles. The membrane hyperpolarization response to NO donors was greatly reduced in W/WV muscles, but muscle relaxation caused by NO donors was of similar magnitude in wild-type and W/WV muscles. Thus different mechanisms appear to mediate nitriergic responses in ICC-IM and smooth muscle cells. The importance of one mechanism might be emphasized in wild-type muscles and compensated for by redundant mechanisms in muscle cells that develop in the absence of ICC-IM. ICC-IM were also greatly reduced in numbers, and inhibitory and excitatory junction potentials were reduced in muscles of Steel mutants (Sl/SlI) that lack membrane-bound stem cell factor (403). Lower esophageal and pyloric sphincter muscles of W/WV mice (408) and small intestinal muscles treated with neutralizing antibodies to c-Kit (407) also displayed reduced populations of ICC-IM (ICC-DMP in small intestinal muscles) and reduced postjunctional responses to stimulation of enteric motor neurons. From these studies, it was reasoned that loss of ICC-IM results in reduced connectivity between enteric motor neurons and smooth muscle cells, and ICC-IM have an important role in transducing motor neurotransmitters in GI muscles.

2. Close contacts between nerves and effector cells in visceral motor neurotransmission

There have been many reports about the close connectivity between enteric motor nerve varicosities and ICC (see sect. IIA; FIGURES 2 AND 3); however, few quantitative studies on this subject have been provided. Clearly, very close (<20 nm) connections with varicosities are not exclusive to ICC, and a few studies (73, 110, 264) have described similar connections between neurons and smooth muscle cells. One of these studies, using a morphometric approach, concluded that close connections were more prevalent between varicosities and ICC (73). A quantitative understanding of the structural features of motor innervation is important, because this may provide spatial parameters necessary for modeling the dynamic profiles of neurotransmitters released from motor neurons. Basic questions regarding enteric motor neurotransmission, such as the nature and molecular specializations of neural active zones (i.e., neurotransmitter release sites), probabilities of vesicle or neurotransmitter release from varicosities per impulse, number of transmitter molecules released upon vesicle fusion, effects of frequency on types and quantities of neurotransmitters released, prejunctional modulation of transmitter release, metabolic and uptake pathways involved in deactivation of neurotransmitter signals, and dynamics of neurotransmitter concentrations in postjunctional volumes are poorly understood (329). Thus discussions about whether “volume” or “synaptic-like” neurotransmission dominate in muscles of the GI tract are speculative at present. Even if volume transmission seems likely from rigorous consideration of morphological details (110), the extent to which effective neurotransmitter concentrations reach...
postjuncti onal receptors depends on the many factors listed above.

3. Expression and function of receptors and effectors of neural responses in interstitial cells

Use of immunohistochemical techniques and isolation of smooth muscle and interstitial cells has allowed evaluations of the expression of receptors and effects of neurotransmitter responses and comparative responsiveness of specific populations of postjunctional cells. In some cases an immunohistochemical approach (i.e., functional immunohistochemistry) can be also useful in determining which cells respond to neurotransmitters released from motor neurons because specific changes in postjunctional signaling proteins (e.g., phosphorylation, generation of second messengers, and protein translocations) can be determined.

Several studies have shown that soluble guanylate cyclase (sGC), the physiological receptor for NO, is highly expressed in ICC. The inhibitory effects of NO and NO-dependent enteric inhibitory neurotransmission in GI muscles are blocked by inhibitors of sGC as shown in several studies by the effects of 1H-(1,2,4)-oxadiazolo-(4,3-b)quinazoline-1-one (ODQ) (106, 164, 429). In the rat small intestine, sGC (β-subunit) is highly expressed in ICC-DMP and colocalized with type 1 cGMP-dependent protein kinase (cGK-1) (324). NO synthase (NOS)-immunoreactive neurons were found adjacent to ICC-DMP. cGK-1 expression was also resolved in type 1 (106, 164, 429). In the rat small intestine, sGC (175, 176). Both ICC-IM and PDGFRα⁺ cells express these proteins. Expression of sGC-β in ICC-IM and PDGFRα⁺ cells was confirmed by immunocytochemistry. NOS neurons were in close association with cells expressing sGC. As in the rat, smooth muscle cells show little or no immunoreactivity for sGC. This observation was controlled by clear expression of sGC in smooth muscle cells of the muscularis mucosae and in blood vessels, confirming that the techniques used were capable of detecting sGC in smooth muscle cells. These authors concluded that sGC expression is low in smooth muscle cells in the tunica muscularis. Functional immunohistochemical studies showed that stimulation of nitergic neurons in GI muscle strips or treatment with NO donors enhanced cGMP in ICC (348, 431).

During muscarinic stimulation, PKC isoforms are translocated from the cytoplasm to the membrane to elicit responses. PKCβ, expressed in the intramuscular ICC of the murine small intestine (ICC-DMP), and immunohistochemistry was performed before and after muscarinic stimulation of small intestinal muscles (398). The antibody chosen for these studies did not recognize cytoplasmic PKCβ, but after exposure to ACh or electric field stimulation of intestinal motor neurons, PKCβ, was detected in ICC-DMP. Translocation was verified by showing a shift in PKCβ, from the soluble to the particulate fractions of extracts of intestinal muscles. Translocation of PKCβ, was also initiated by phorbol ester, and muscarinic responses and neurally evoked translocation were blocked by atropine or TTX. These experiments directly demonstrated innervation of ICC-DMP by cholinergic neurons.

ICC-DMP also express neurokinin 1 (NK1) receptors (127, 178, 357, 361), and binding of agonists to these receptors on ICC causes internalization (237). NK1 antibodies were used to test whether membrane internalization could be elicited by stimulation of enteric motor neurons (178). Excitatory motor neurons, which release both ACh and tachykinins in the GI tract, were shown to be closely associated with ICC-DMP expressing NK1 receptors. Stimulation of muscle strips with either exogenous substance P or by electrical field stimulation led to a shift from diffuse immunolabeling of NK1 receptors around the periphery of cells to a pattern of internalized receptors associated with small granular structures within cells after stimulation. Internalization of NK1 receptors in response to field stimulation was blocked by a selective NK1 antagonist (WIN62577) or TTX.

Internalization of NK1 receptors was also exploited as a means to separate ICC-DMP from ICC-MY in the small intestine. Muscles were incubated with substance P labeled with Oregon green (OG-488) before dispersing cells. Cells were sorted for c-Kit and then again for Oregon green. Cells with both labels were considered to be ICC-DMP, as confirmed by fluorescence microscopy. The cells were probed for numerous genes known to be involved in mediation of postjunctional responses to enteric motor neurotransmitters. Genes including Chm2 and Chm3 (cholinergic responses), Tacr1 (neurokinin responses), Gucy1a3 and Gucy1b3 (nitergic responses), P2ry1 and P2ry4 (purinergic responses), and Vipr2 (responses to vasoactive intestinal peptide) were expressed by ICC-DMP. Further work is necessary to determine whether receptor expression by intramuscular ICC in various regions of the GI tract is linked to ion channels and other effectors that contribute to motor nerve responses.

ACh released from motor neurons in the gastric fundus activates different postjuncti onal mechanisms than are activated by muscarinic agonists added to solutions bathing muscles (33). In this study phosphoproteins involved in Ca²⁺ sensitization mechanisms were analyzed before and after addition of carbachol to bathing solutions or after stimulation of cholinergic motor neurons. Neural release of ACh activated only phosphorylation of protein kinase C (PKC)-potentiated phosphatase inhibitor protein (CPI-17), but bath application of carbachol activated phosphorylation of CPI-17 and myosin phosphatase targeting subunit 1 (MYPT1). When acetylcholine esterase was inhibited by...
neostigmine, activation of cholinergic neurons caused phosphorylation of CPI-17 and MYPT1. Stimulation of cholinergic neurons in W/WV mice also caused phosphorylation of CPI-17 and MYPT1. These data suggested that ACh released from neurons is compartmentalized, possibly within the tiny volumes created by the close apposition of nerve varicosities and ICC. Due to this compartmentalization, ACh released from neurons does not appear to reach smooth muscle receptors linked to activation of Rho kinase and MYPT1 phosphorylation. Inhibiting ACh metabolism or reducing contacts between ICC and motor neurons and the small junctional volumes formed as a result of these contacts appears to enhance the availability of ACh and exposure of the neurotransmitter to smooth muscle receptors.

Taken together, there is significant evidence suggesting direct innervation of ICC by enteric motor neurons. Studies summarized above show spatial proximity of ICC to nerve varicosities, loss of postjunctional responses in the absence of ICC, expression of receptors for enteric motor neurotransmitters, translocation of signaling molecules, and internalization of receptors in ICC upon nerve stimulation. Of course, parallel neurotransmission to other SIP syncytium cells is quite possible; smooth muscle cells and PDGFRα+ cells also lie in close proximity to varicosities of motor neurons. The involvement of PDGFRα+ cells will be discussed below. At this point it seems important to note that there is little evidence in the literature of direct motor innervation of smooth muscle cells before removal of ICC.

4. Studies reporting normal neuromuscular responses in W mutants

There are reports in the literature demonstrating sustained responses to nitricergic and cholinergic neurotransmission in W/WV mice and Ws/Ws rats (165, 432). These findings have led some authors to conclude that ICC are not important or necessary for enteric motor neurotransmission. However, a better question to ask might be whether responses to neurotransmitters and other bioagonists are “normal” in the absence of ICC. Even though contractile responses to neurotransmitters are apparent in the absence of ICC, it is clear that responses differ from wild-type responses. In the case of the gastric fundus, loss of ICC led to recruitment of a Ca2+ sensitization mechanism not normally activated by ACh released from neurons (33), thus increasing the gain on smooth muscle contractile responses. This may help preserve (or even amplify) cholinergic contractile responses in muscles lacking ICC-IM, but it would also tend to alter responses to all other physiological agonists, such as paracrine substances and hormones. Such an effect might adversely affect integrated motor responses in the stomach.

Ws/Ws rat fundus muscles developed significantly less spontaneous tone than wild-type rats, and responses to nerve stimulation were variable (165). Nerve stimulation resulted in contraction or relaxation under circumstances in which relaxation responses were elicited in wild-type muscles. Tone developed spontaneously in wild-type muscles, but phasic contractions were noted in W mutants. Noncholinergic excitatory responses appeared elevated in Ws/Ws rat muscles. Differences in motor patterns and variability in the dominance of excitatory or inhibitory regulation in different animals indicate remodeling of neuromuscular responses in animals with mutant W alleles.

Postjunctional nitricergic responses were also reported to be normal, or at least preserved, in a study of neuromuscular responses in the lower esophageal sphincter (LES) of W/WV mice (432). Some animals displayed nitricergic responses while muscles of other animals of the same genotype did not. It was also reported that ICC-IM were retained in some regions of the LES; however, the presence or absence of cells labeled with c-Kit+ antibodies did not appear to correlate with nitricergic responses. Although some muscles displayed nitricergic components during inhibitory junction potentials, most of the electrical responses displayed in this paper (e.g., see FIGS. 3–6 of Ref. 432) were similar to the responses of LES muscles from W/WV mice (e.g., reduced nitricergic components of IJPs and absence of membrane STDs) that had been described previously (408).

In other studies, responses monitored by manometry were compared in the LES of W/WV and nNOS−/− mice (352). These authors reasoned that if ICC-IM are required for nitricergic responses, then pressure responses in the LES of W/WV and nNOS−/− mice should be very similar. However, the motor behaviors of W/WV and nNOS−/− mice were different: tone was elevated in the LES of nNOS−/− mice and relaxation responses were abnormal; tone was greatly depressed in W/WV mice and relaxation responses were considered normal. This study seems to have neglected previous reports showing that ICC-IM are involved in mediating responses to excitatory and inhibitory neural inputs (45, 400). Therefore, one might predict that responses in W/WV and nNOS−/− mice would be quite different. W/WV mice would be expected to have reduced LES tone due to reduced cholinergic input. If only nitricergic inputs are compromised, as in nNOS−/− mice, LES tone may be elevated and relaxation responses would be greatly decreased. It should also be noted that it is problematic to compare the magnitudes of responses in muscles with different levels of basal tone, and the conclusions that nitricergic responses were normal in the LES and pyloric sphincter of W/WV mice were based on such comparisons (351, 352).

Lesions in ICC are not homogeneous throughout the bowel in W mutants (45, 404). At present, the relationship between loss of ICC and loss of motor function has not been quantified, and in the case of inhibitory regulation of contractions, the relationship might be very complicated. For example, regulation of contraction by membrane potential...
is highly nonlinear and might be accomplished in the gut by small membrane potential changes. Membrane potentials of GI muscles, at rest in tonic muscles or at the peaks of slow waves in phasic muscles, lie near or within the range of potentials in which the open probability of L-type Ca$^{2+}$ channels is steeply dependent on voltage. Therefore, small hyperpolarization responses can greatly reduce the open probability of Ca$^{2+}$ channels and reduce Ca$^{2+}$ entry to levels that do not sustain contraction. In fact, typical hyperpolarization responses in wild-type muscles take membrane potential far negative to the range in which the open probability of Ca$^{2+}$ channels is sufficient to elicit contraction. Therefore, hyperpolarization responses might be greatly attenuated before contractile responses are inhibited. Regions of the gut with reduced ICC or reduced ICC function might not show motor effects without a substantial reduction in ICC. Studies have been conducted on muscles of W mutants from regions of the gut with partial reduction in ICC. For example, ICC were reduced but not abolished in colon of Ws/Ws rats (4). Nitrergic components of IJPs were recorded from approximately half of cells impaled for electrical recording, and nitrergic responses were reduced or not resolved in the remaining cells. Tabulation of ICC at or near each impalement was not provided, so it is unknown whether loss-of-function correlated with a localized reduction in ICC. Other studies of this type were reviewed previously (329). It should also be noted that within a given region of muscle (e.g., gastric fundus) there is variation in the numbers of c-Kit$^{+}$ cells from animal to animal. Thus studies in the future using W mutants should include assessments of ICC populations in regions from which neural responses are recorded.

5. Cell specific inactivation of neurotransmitter receptors or effectors

Two recent reports have utilized the Cre/loxP recombination approach to target activation or deactivation of specific genes in ICC. c-KitCreERT2 mice were generated to target inducible activation of iCre recombinase in ICC (200). By crossing these mice with a strain with conditional expression of diphtheria toxin A, it was possible to reduce ICC in adult mice. Depletion of ICC caused significant disturbances in GI motility. Membrane potentials of small intestinal muscles were depolarized, slow wave activity was disrupted, and there were significant slowing of gastric emptying and intestinal transit. Mice with disrupted ICC exhibited no excitatory or inhibitory junction potentials in the small intestine, suggesting that reducing the number of ICC effectively denervated the smooth muscle. In the colon, excitatory junction potentials were greatly reduced by loss of ICC, but effects on inhibitory junction potentials were less affected. Targeted deletion of Prkg1, which encodes a major effector for nitrergic neurotransmission, PKG1, caused significant reduction in nitrergic responses and delayed colonic transit. In another study using ICC and smooth muscle specific iCre strains, the β1 subunit of guanylate cyclase (Gucy1b3) was targeted in ICC, smooth muscle cells or in both types of cells (131). Knocking down soluble guanylate cyclase (sGC) in fundus smooth muscle cells caused reduction in responses to a bath-applied NO donor, but responses were not greatly affected with knockdown of sGC in ICC. Responses to NO released from neurons was not greatly affected in mice with cell-specific knockdown of sGC in smooth muscle cells or in ICC, but knocking down sGC in both cell types blocked most of the nitrergic response in the fundus. These authors attributed nitrergic responses, therefore, to combined effects on ICC and smooth muscle cells, suggesting parallel neurotransmission to both cell types.

Animal models in which specific genes can be inactivated selectively in one type of cell will be powerful tools for future exploration of the role of interstitial cells in enteric motor functions. One must be cautious in interpreting the responses of iCre-dependent gene inactivation models, however, because this approach seldom results in quantitative inactivation of floxed alleles, particularly when crosses are made with homozygous mice with two floxed alleles in the targeted cells (15). In such cases, recombination of both floxed alleles must occur for knockout of gene transcription in a given cell. Leaving one functional allele results in partial depletion of gene products, as in heterozygotes. It is well known that heterozygotes often display functions equivalent to the wild-type and are frequently used as control animals in gene inactivation studies. Assessment of the degree of gene inactivation in the cells targeted for expression of Cre or iCre is important information to include in future studies using this technology.

6. PDGFRα$^{+}$ cells in enteric motor neurotransmission

ICC are not the only cells in the postjunctional environment that respond to enteric motor neurotransmitters. PDGFRα$^{+}$ cells are also closely associated with varicosities of motor neurons and intermingled with ICC around nerve processes (174, 177, 221). Isolation of colonic PDGFRα$^{+}$ cells from Pdgfra$^{tm11(EGFP)Sor}$, a reporter strain with eGFP expressed in cell nuclei driven off the endogenous Pdgfra promoter (135), allowed positive selection of cells for molecular and functional studies (13, 221). Cells from colon and stomach sorted by FACS displayed robust outward currents (STOCs) that were blocked by apamin. Sin-
purines, such as ATP, β-nicotinamide adenine dinucleotide (β-NAD), and ADP, activated Ca²⁺-dependent K⁺ currents under voltage clamp, and these responses were blocked by apamin and the P2Y1 receptor antagonist MRS2500 (221). In current-clamp mode, purines activated rapid responses that hyperpolarized cells to E_K. In contrast, responses of colonic smooth muscle cells held at physiological resting potentials were minimal, or small inward currents were activated. Smooth muscle cells had small depolarization responses to ATP in current clamp. These results show that PDGFRα+ cells mimic the purinergic neural responses of whole colonic muscles (i.e., hyperpolarizations inhibited by apamin and MRS2500; Refs. 116, 173), and these responses do not occur in smooth muscle cells at physiological potentials. ICC also show little or no response to purines that can explain purinergic responses in situ. Thus PDGFRα+ cells are the most likely postjunctional cells to mediate purinergic motor neurotransmission. PDGFRα+ cells may also contribute to tonic inhibition in the colon. PDGFRα+ cells generate STOCs, and this could provide a net stabilizing effect on the excitability of smooth muscle cells. It should also be noted that PDGFRα+ cells express sGC (175, 176), but a role for NO/cGMP-dependent mechanisms in these cells has not been identified.

Activation of SK3 currents in PDGFRα+ cells would require dynamic cytoplasmic Ca²⁺ signaling. The occurrence of STOCs in these cells suggests that periodic, localized Ca²⁺ release from intracellular stores is an important mechanism. Ca²⁺ signaling mechanisms were investigated in PDGFRα+ cells of gastric fundus muscles in situ (13). Cells in fundus muscles of Pdgfra<sup>+/EGFP</sup> mice were loaded with fluo 4 and identified by nuclear eGFP. Spontaneous localized Ca²⁺ transients were observed in PDGFRα+ cells. Purines (ATP, ADP, UTP, and β-NAD) and the P2Y1 agonist MRS-2365 enhanced Ca²⁺ transients, and these responses were blocked by MRS-2500, a specific P2Y1 antagonist. ADP, MRS-2365, and β-NAD failed to elicit Ca²⁺ transients in cells of P2ry<sub>1−/−</sub> mice, but responses to ATP persisted. Phospholipase C (U-73122), IP₃ receptor (2-APB), ryanodine receptor (ryanodine), and SERCA pump (cyclopiazonic acid and thapsigargin) inhibitors blocked Ca²⁺ transients evoked by purines. Activation and regulation of Ca²⁺ transients are likely to mediate the openings of SK3 channels, generation of STOCs and responses to purines, and purinergic inhibitory junction potentials in GI muscles.

7. Neuromuscular transmission and integration in the SIP syncytium

As discussed above, the extent to which motor neurotransmission is “volume-like” or more focalized (e.g., synaptic-like due to close appositions between varicosities and postjunctional cells or restrictions on transmitter diffusion due to metabolism or uptake) is controversial at present. What seems not debatable, based on current knowledge, is that at least three postjunctional cell types are clustered within 100 nm of nerve varicosities in GI muscles, and each of these cells expresses specialized receptors and effectors that respond to enteric motor neurotransmitters. Binding of neurotransmitter molecules to ICC and PDGFRα+ cell receptors activate or modulate ionic conductances, as described above, and these cells are electrically coupled to smooth muscle cells by gap junctions. Thus the neuroeffector junction in GI muscles is more accurately conceptualized as the SIP syncytium, composed of electrically coupled smooth muscle cells, ICC, and PDGFRα+ cells (FIGURE 3) (209, 330). Changes in the conductance of any type of SIP cell can affect the input resistance of the syncytium. Together SIP cells not only determine the integrated excitability of muscle layers and responses to neurotransmitters, but it is quite likely that responses to hormones and paracrine mediators are also integrated through the responses of the SIP syncytium. Classically, terms such as “myogenic,” “neurogenic,” and “neuroeffector junction” used to describe different regulatory mechanisms in GI tissues have not been inclusive of the important responses elicited by interstitial cells.

G. ICC as Stretch Receptors in GI Muscles

The walls of GI organs go through dramatic distortions while filling and during the digestion and movement of food and wastes. Stretch-dependent mechanisms have been detected in neurons (84, 219, 358, 359) and smooth muscle cells (97), but interstitial cells may also have an important “sensory” role in transducing mechanical events and providing immediate feedback affecting motility. Freshly isolated ICC from human intestine displayed a Na⁺ current in patch-clamp experiments (362). The conductance was blocked by QX-314 but not by nifedipine or Ni²⁺. Transcripts for SCN5A, which encode α subunits of cardiac TTX-resistant Na⁺ channels, were found by single-cell PCR. Stretch of intestinal muscle increased the frequency of slow waves, and QX-314 or lidocaine reduced slow wave frequency. These data suggest that pacemaker ICC express a mechanosensitive Na⁺ conductance that regulates slow wave frequency.

Another study investigated the chronotropic effects of stretch in the murine gastric antrum (417). Slow waves were recorded from antral muscles during computerized application of muscle stretch at a rate of 6.0 μm/s to a maximum tension of 5 mN. This increased muscle length by ~27%, which is within what might be expected physiologically during antral contractions. Stretch of antral muscles caused 4 mV of depolarization and increased slow wave frequency from 2.5 to 3.6 cpm. The magnitude of the response to stretch was positively related to the rate of muscle stretch. Stretch responses were blocked by reduced extracellular Ca²⁺ and by Ni²⁺, but unaffected by nifedipine. Responses to stretch were not neurogenic because they were not blocked by TTX. Acceleration of antral slow waves dis-
ruptured corpus to antrum slow wave propagation temporarily. Stretch responses were not recorded in muscles of \( W/W^{v} \) mice, suggesting that ICC-IM may mediate stretch-dependent chronotropic effects. It was also found that indomethacin blocked stretch-dependent chronotropic responses, and these responses were also not present in mice lacking cyclooxygenase-2 (\( \text{Pgs2}^{-/-} \)). These findings suggest that stretch may activate phospholipase A\(_{2} \) and generate arachidonic acid. Synthesis of PGE\(_{2} \), a product of COX-2, may drive stretch-dependent responses in ICC-IM, since this prostanooid has been shown to have positive chronotropic effects in antral muscles of several species (103, 327, 333).

**H. Interactions With Afferent Nerve Fibers**

There are at least two types of mechanoreceptor structures arising from extrinsic afferent neurons in smooth muscles of GI organs: intraganglionic laminar endings (IGLEs) and intramuscular arrays (IMAs). The morphology and intercellular associations of IMAs have been carefully described in recent years; however, the function of these structures is still poorly understood (294). IMAs arborize into a complex array of terminal processes that run in parallel to the smooth muscle fibers and are associated with ICC-IM in the stomach (32, 295). IMAs are also associated with three species of efferent fibers, distinguished by antibodies to nNOS (inhibitory motor neurons), vesicular acetylcholine transporter (excitatory motor neurons), and tyrosine hydroxylase (sympathetic neurons). Calcitonin gene-related peptide (CGRP)-immunopositive afferent fibers (presumed to be collaterals of visceral afferent fibers) are also associated with IMAs (294). The varicosities of IMAs form synaptic-like contacts with ICC and display vesicles and prejunctional densities (166, 294). The ICC-IMA complex has been likened to a functional correlate of the muscle spindle organ of striated muscles. The function of ICC in relation to IMAs has not been clarified. There is evidence that vagal afferents can engage in axon reflexes (414), which is accomplished by branches of peripheral axons that contain both sensory and effector functions without the benefit of any synapses or an integration center. In the case of ICC-IMA complexes, the numerous lamellar varicosities of IMAs provide afferent limb of intrinsic GI reflexes (109). IMAs are proposed to have a characteristic electrophysiology signature of “after-hyperpolarization” (AH) following action potentials. Therefore, IPANs are also known as AH neurons. Identified AH neurons were described as making close contacts with ICC-MY, and depolarization of the neurons correlated with modulation of Ca\(^{2+} \) transients in ICC-MY (438). These authors concluded that direct inputs from AH neurons regulate ICC-MY function.

**III. DEVELOPMENT AND PLASTICITY OF ICC**

**A. Role of c-Kit in Development of ICC Networks**

ICC are of mesodermal origin (238, 379, 430) and derived from cells within the gut. Cells expressing transcripts of \( \text{Kit} \) appear on about embryonic day 9 (E9) in mice (280), and c-Kit protein expression is detected by immunohistochemistry at E12 (379). Initially c-Kit\(^{+} \) cells form a layer of undifferentiated mesenchymal cells around the periphery of the intestine (at E12). c-Ret\(^{+} \) cells, which are precursors of enteric neurons and glia, form a distinct layer at the inner surface of the c-Kit\(^{+} \) cells. Circular muscle cells are apparent at E15, but at this stage the intestine lacks a longitudinal muscle layer. Within the circular muscle layer, smooth muscle antigens, such as \( \gamma \) enteric actin and desmin, are prominent, and the cells have typical ultrastructural features such as thin and thick myofilaments, intermediate filaments, dense bodies, caveolae, and well-developed endoplasmic reticula. c-Kit\(^{+} \) cells are largely immunopositive for vimentin, but a few cells begin to develop immunoreactivity for desmin at E15, suggesting that cells of the longitudinal muscle layer may develop from c-Kit\(^{+} \) precursors (379). Others reached a similar conclusion by showing with in situ hybridization that cells at the periphery of the small intestine that expressed \( \text{Kit} \) mRNA coexpressed myosin heavy chain transcripts (203). By E18, a clear longitudinal muscle layer is formed and c-Kit\(^{+} \) cells developed processes and formed a lose network in the region of the myenteric plexus (ICC-MY). Longitudinal muscle cells express desmin, and ICC retain expression of vimentin. The other population of ICC in the small bowel (ICC-DMP) is not found in murine embryos and develops within the first week after birth.

The heterogeneity of ICC lesions in \( W/W^{v} \) mice (404) led to speculation that c-Kit is not required for development of all ICC. Some populations of ICC fail to develop with the loss of function in \( W/W^{v} \) mutants, but other populations of ICC develop, albeit usually in reduced density and with reduced immunoreactivity to c-Kit antibodies. These observations suggest that ICC from different regions of the GI tract and even within specific regions have different sensitivities to
c-Kit signaling. Another possible difference might be in survival of different ICC in the absence of c-Kit. One study investigated the development of ICC in animals with Kit alleles deactivated by knock-in of LacZ into the Kit locus. Homozygotes for the transgene (Kit<sup>lacZ<sup>+/+) expressed c-Kit and ICC normally, but Kit<sup>LacZ/LacZ mice lacked c-Kit but cells identified as ICC were as abundant as in Kit<sup>LacZ<sup>+/+ mice (31). It is possible, however, that the cells identified as ICC were precursors for ICC identified in development by E15 (379). Whether the cells identified as ICC (at about E18) were functional was not determined. Another group investigated the distribution of ICC in Wbd<sup>T(wbanded) mice (203). Wbd contains a genomic rearrangement of chromosome 5 that results in inversion of the c-kit gene and ectopic expression of Kit (204). These mice displayed normal distributions of cells identified as ICC at D5, but adult animals lacked ICC. The authors concluded that ICC did not require signaling via c-Kit for development, but functional c-Kit was required for maintenance of the ICC phenotype as animals matured. Identification of cells as ICC was performed by methylene blue staining, but there was no verification that the cells labeled with methylene blue were ICC. Electrical recordings failed to demonstrate slow wave activity in Wbd<sup>/ Wbd mice, suggesting that the ICC pacemaker phenotype never developed. Thus the question is whether ICC developed in Wbd<sup>/Wbd<sup> mice or whether another cell type, labeled by methylene blue, was identified as ICC in these mutants. For example, it has never been determined whether methylene blue labels ICC exclusively or whether PDGFRα<sup>+ cells are also labeled by this dye.

The question of the role of c-Kit in development of functional ICC during the late embryonic period was addressed in another study by treating muscles with neutralizing c-Kit antibodies from E15 through birth. Functional ICC develop in small intestinal segments in organotypic cultures started at E15; however, inclusion of c-Kit neutralizing antibodies blocked development of c-Kit positive cells and pacemaker activity (406). It was also observed that ICC-MY and slow waves developed normally in W<sup>V/+ and in W<sup>/ hyperoxygenates, but failed to develop between E17 and birth (postnatal day 0; P0) in W/W<sup>V embryos (23). Similar results, including loss of ICC and failure of pacemaker development, occurred in muscles treated for several days with the tyrosine kinase inhibitor imatinib mesylate (STI157). These data support the concept that ICC precursors cells might emerge in animals with compromised c-Kit function, but subsequent development into mature and functional ICC requires c-Kit signaling.

The reasons why ICC develop within specific planes in the tunica muscularis (e.g., ICC-MY and ICC-SM) or in association with specific structures, such as enteric neurons (ICC-DMP or ICC-IM), are poorly understood, but the patterns of ICC localization are conserved, with minor variations, in mammalian species and fish (14, 210, 307). ICC require signaling via c-Kit for development (23, 306, 404), so one explanation for their localization within muscles might be that they are concentrated near cells expressing stem cell factor, the ligand for c-Kit. Experiments on transgenic animals with lacZ linked to the promoter for stem cell factor showed that enteric neurons express stem cell factor (380), but others have reported that smooth muscle cells also express stem cell factor transcripts and protein (161, 238, 422). Expression of stem cell factor by neurons might direct positioning of ICC, because both nerve cell bodies or processes occupy most of the same niches as ICC. However, it is unclear how smooth muscle expression of stem cell factor might direct the localization of ICC with such precise patterning. In fact, experimental evidence suggests that neurons, and thus trophic factors expressed by neurons, are not obligatory for development or localization of ICC, because ICC developed in muscles taken for culture from mammalian and bird intestines before colonization of the muscles with neural crest cells (238, 430). Functional ICC also developed in c-Ret<sup>−/− mice that lack enteric neurons below the proximal stomach (409). Therefore, significant questions remain about the signals and sources of signals directing the positioning, architecture, and connectivity of interstitial cell networks.

Significant development of ICC occurs in mice during the postnatal period. At P0, ICC-MY form a loose network with cells displaying a few short and branching processes. The density of ICC-MY at P0 was ~750 mm<sup>−2. The demands of maintaining the structure of ICC networks as bowel diameter expands require proliferation of ICC. An age-dependent proliferation of ICC-MY, estimated to be ~15-fold from neonates to adulthood, maintained the relative density of these cells in adult animals (259). The proliferative potential of ICC was studied by incorporation of 5-bromo-2'-deoxyuridine (BrdU). A large number of cells within ICC networks were labeled with BrdU shortly after birth, but the number of cells incorporating BrdU, 24 h after loading, decreased as a function of age. Cells with short processes, but apparently already integrated into ICC-MY networks, incorporated BrdU within 1 h after injections, and pairs of cells with BrdU were noted after 24 h. It was difficult to find proliferating cells after P24. The fraction of total cells that incorporated BrdU was also determined by daily injections, and this number increased to 64% by P24. The expansion of ICC-MY appeared to be due to proliferating cells within ICC networks (259), as opposed to proliferation from a rare population of Kit<sup>lowCD44<sup> CD34<sup>−IGF-1<sup>− cells, proposed by others as the progenitors of ICC (248). It should be noted that most cells within the ICC-MY network were CD44<sup>+, and some cells showed weak labeling with antibodies to CD34 and insulin-like growth factor 1 (IGF-I) (259). These findings tend to suggest that Kit<sup>−CD44<sup> CD34<sup>−IGF-1<sup>− cells are normal components of ICC-MY networks and do not necessarily represent a specialized population of ICC progenitors.
Early in human development (7 wk), c-Kit+ cells can be found along the entire length of the esophagus in the region of the myenteric plexus, but this distribution changes with age such that ICC were far more abundant in the distal one-third versus the proximal esophagus by weeks 13–16 (300). After 20 wk, ICC were extremely rare in the proximal (skeletal muscle) portion of the esophagus and had developed into spindle-shaped ICC-IM in the circular and longitudinal muscle layers in the distal third (smooth muscle) portion of the esophagus. The middle third of the esophagus displayed ICC in septa between smooth and skeletal muscle fibers. ICC developed over approximately the same window of time in the stomach, forming first a layer of c-Kit+ precursors around the periphery of the organ adjacent to the developing myenteric plexus (301). By 20 wk, ICC can be found in all of the adult niches: ICC-MY, ICC-IM, and ICC-SEP. ICC develop in the human midgut and hindgut somewhat later than in the proximal GI tract. At 9 wk, c-Kit labeling is present at low levels within the circular muscle (393). By week 11, c-Kit labeling is present along the inner and outer aspects of myenteric ganglia. At 14 wk, ICC displayed an approximately adultlike distribution. In the distal colon, c-Kit+ cells emerged during weeks 10–11. Two parallel bands of cells were found within the myenteric region between the circular and longitudinal muscle layers and near the submucosal edge of the circular muscle (299). The proximal to distal delay in the development of ICC in the human GI tract is also a feature of the development of ICC and pacemaker activity in the mouse (379, 406).

B. Development of Electrical Rhythmicity in ICC

A lineage decision is made at about E15 in the murine intestine, and cells that continue to express c-Kit develop pacemaker activity (379). Electrophysiological studies of jejunal muscles during the late embryonic period found no electrical slow waves at E17, but small-amplitude fluctuations in membrane potentials (STDs) were observed. Networks of ICC were well-articulated at E17, and multiple processes linking cells together were observed (23, 379, 406). At E18, slow waves, resistant to block by dihydropyridines (a signature feature of slow waves vs. action potentials), were recorded by intracellular microelectrode techniques (23). The organization, frequency, and amplitude of slow waves continued to develop through birth and the first week after birth, reaching mature amplitude and frequencies by about P10. Other investigators set the onset of electrical rhythmicity in the proximal small intestine later in development, finding weak electrical activity in unfed neonates and strong reduction in the electrical activity by verapamil (246). These authors concluded that ICC-MY were not functional at birth, and pacemaker activity and slow waves did not develop until about P2. ICC-MY were identified with methylene blue in this study, but this dye is less reliable than immunohistochemical techniques (see sect. IIA1).

Consistent with the role of ICC as pacemakers, there is a proximal-to-distal delay in the developmental of pacemaker activity (406). Slow waves were recorded from the proximal GI tract (stomach and antrum) during the late embryonic period (as described above), but developed after birth in the ileum and colon. Slow wave activity with clusters of action potentials superimposed, characteristic of the proximal colon, developed in colonic muscles by P6–P8; however, this activity was infrequent, with long periods of quiescence between events. Regular, rhythmic slow waves and action potential clusters were not fully developed in the proximal colon until about P10.

Development of ICC and slow waves occurs unfettered in organotypic cultures of GI muscles (23, 406), and this approach has been utilized to correlate the timing of ICC-MY development and the development of electrical rhythmicity and to evaluate the impact of c-Kit signaling on development. Inclusion of neutralizing c-Kit antibodies in muscles of neonatal animals abolished ICC networks and blocked pacemaker activity within a few days of exposure. At birth, as observed in animals, ICC were much more susceptible to effects of neutralizing c-Kit antibodies than after developing in animals for several days before being put into organ culture (406). Longer periods (e.g., >30 days) of exposure to c-Kit antibodies were required to lesion ICC-MY networks and block pacemaker activity in muscles from mature (>P20) animals. Since the dependence on c-Kit signaling for initial development of ICC-MY was challenged by others (31, 203), experiments were also performed to determine whether functional ICC developed with c-Kit blocked or disabled genetically. ICC-MY and slow waves developed in jejunal muscles of wild-type, W/+, and Wv/+ mice by E18, but failed to develop in muscles of W/Wv mice. ICC-MY failed to develop in jejunal muscles removed from mice at E17 (i.e., before slow waves develop) and cultured with neutralizing c-Kit antibodies (23). Similar results were obtained if muscles were put into organ culture after slow waves developed (E18-P0). Imatinib mesylate (STI157), a c-Kit inhibitor, had no acute effect on slow waves, but exposure to this compound blocked postnatal growth and development of ICC-MY and slow waves. Thus c-Kit signaling during development is requisite for establishment and maintenance of the pacemaker function of ICC.

C. Fate of ICC When c-Kit Is Blocked

Loss of ICC has been observed in human GI muscles from patients with a variety of motility disorders (11, 43, 143, 168, 205, 385). Dysmotilities seem an obvious consequence of defective ICC or loss of ICC due to the important functions of these cells. In spite of the clinical significance, the factors responsible for ICC loss in patients are poorly un-
derstood. Attempts have been made to develop animal models of ICC loss with the idea of establishing the cause and effect of ICC loss in motility disorders and to study the plasticity of ICC.

ICC are vulnerable to block of c-Kit signaling in neonatal and adult animals; however, the time required for c-Kit block increases from the postnatal period to adulthood. Neutralizing c-Kit antibodies injected into neonatal mice and for several days after birth cause loss of ICC within a week (250, 378), and all classes of ICC are affected. The c-Kit inhibitor imatinib mesylate also lesions ICC networks in neonatal murine intestinal muscles in organ culture (23). In adult guinea pigs, imatinib caused a time-dependent reduction in ICC-MY of the small intestine, shortening of cell processes, and generalized thinning of the network (258). The number of ICC-MY was reduced by about one-third after 16 days of treatment. ICC-DMP were also affected, but effects were less dramatic and slower in onset. No evidence of apoptosis was noted in ICC after treatment with imatinib; instead, remaining cells coexpressed α-smooth muscle actin, suggesting that ICC-MY undergo a phenotypic change toward a smooth muscle or myofibroblast phenotype when c-Kit is blocked. BrDU incorporation was observed in ICC-MY after withdrawal of imatinib, and pairs of cells with highly developed processes that were well-incorporated in ICC-MY networks demonstrated that cell division of mature ICC was at least partially responsible for repopulation of ICC-MY networks after ending c-Kit blockade (258).

Downstream cell signaling initiated by stem cell factor binding to c-Kit includes contributions from phosphatidylinositol 3’-kinase (PI3-kinase), phospholipase C-γ (PLC-γ), phospholipase D, SRC family kinases, p21ras GTPase-activating protein, and mitogen-activated protein kinase (MAPK) (244). There are multiple classes of PI3-kinase, but growth factor receptors typically activate class Iα. Binding of stem cell factor leads to autophosphorylation and dimerization of c-Kit and generation of high-affinity binding sites for signaling molecules, including PI3-kinase. Phosphorylation of c-Kit leads to binding of the p85 regulatory subunit of PI3-kinase and subsequent activation of the catalytic subunit p110. The effects of PI3-kinase inhibitors, wortmannin and LY 294002, were tested on development of ICC and pacemaker activity in mice. The amplitude of slow waves increases after birth, and this development also occurs in organotypic cultures (406). Inclusion of wortmannin or LY 294002 in organ cultures of intestinal muscles taken from animals at birth caused loss of ICC-MY and blocked slow waves within a few days (401). Pacemaker activity was also blocked in adult muscles by PI3-kinase inhibitors, but up to a month of exposure was required to develop these effects. Another study employed a transgenic mouse with a tyrosine 719 to phenylalanine (Y719F) mutation in Kit (119). No loss of ICC function was observed in these mice, and it was concluded that signaling through PI3-kinase is not required for development and function of ICC. There are two means of activating PI3-kinase, however, by direct binding of PI-3kinase to the phosphorylated tyrosine residue of c-Kit and indirectly by binding to the tyrosine phosphorylated accessory protein GAB2 (271). Thus the Y719F mutation in Kit may not block all PI3-kinase signaling.

ICC-MY networks and slow wave activity can be reestablished after removal of conditions or reagents that block c-Kit. This point will be more completely illustrated in the next section, but model studies of this concept have been performed on muscle strips in organ culture. As above, treatment of P0 muscles with neutralizing c-Kit antibodies or imatinib mesylate for 3 days caused loss or dramatic reduction in ICC-MY and loss of pacemaker activity. After removal of these reagents, ICC networks and slow waves were restored within 9 days (23).

D. Animal Models of ICC Loss and Pathophysiology

In addition to Kit mutants and animals in which c-Kit is blocked, ICC loss has been reported in several animal models of GI motility disorders. These are important because parallel examples of ICC loss occur in similar or equivalent diseases in human patients. It should be noted that if a common factor is responsible for ICC loss in various disease states, it has not been identified. Furthermore, a debate exists about whether functional ICC are lost through cell death or by remodeling of the ICC phenotype. Examples of animal models with predictable, and in some cases reversal, defects in ICC are described below.

1. Bowel obstruction

Partial obstruction of the bowel leads to marked distension, hypertrophy, and hyperplasia of smooth muscle cells, and thickening of the muscle layers of the bowel proximal to the obstruction (111, 112). Studies of a rat model of partial obstruction showed remodeling of enteric neurons and reduced numbers of ICC in the hypertrophic region of bowel (91). In mice, partial obstruction resulted in hypertrophy in a long segment of small intestine and a lesion in ICC networks that was nearly complete a few millimeters above the obstruction but decreased in severity over 100 mm of bowel (51). In specific planes of the tunica muscularis normally occupied by ICC, cells with ultrastructural features more typical of fibroblasts were observed, but there was no evidence of cell death or necrosis (e.g., shrinking, condensed or fragmented nuclei, indicative of apoptosis, or cell swelling or bursting) was observed in any region where ICC were lost. Slow waves were greatly reduced in amplitude or absent in the section of bowel with the most extensive loss of ICC and responses to neural inputs were also greatly re-
duced in these regions. ICC, slow wave generation, and neural responses recovered in ~1 mo if the obstruction was relieved.

2. Diabetes

Reduction in ICC in diabetes and the importance of this phenomenon in diabetic gastroparesis has been a topic of interest since defects in ICC networks were noted in a mouse model of type I diabetes. Non-obese diabetic mice (NOD/LtJ) mice were shown to have delayed gastric emptying and reduced and arrhythmic slow wave activity in the antrum (278). Fundus muscles from NOD/LtJ mice were less responsive to activation by enteric motor neurons, which might be associated with reduced gastric accommodation during ingestion of food. Functional defects were associated with reduced ICC in the antrum and loss of close connectivity between motor neurons and ICC in the fundus. Another study confirmed the link between loss of ICC and development of delayed gastric emptying, and showed that loss of Kit expression, the marker for ICC, was the most consistent correlate for defects in gastric emptying (57). These authors noted that hemeoxygenase-1 (HO1) expression, which normally is expressed at low levels in the tunica muscularis, increased as diabetes developed. Mice that maintained expression of HO1 were protected from development of delayed gastric emptying, and mice that failed to maintain levels of HO1 developed emptying delays. It was suggested that the benefit of HO1 in diabetes may be to protect ICC against enhanced oxidative stress. An exciting finding was that induction of HO1 expression by hemin rescued mice that had developed delayed gastric emptying. Resident macrophages, which lie in close proximity to ICC, were identified as the cells with enhanced expression of HO1 in diabetes. The protective effects of HO1 on gastric emptying appear to be due to the production of carbon monoxide (CO) (190). Inhalation of CO by NOD/LtJ mice with delayed gastric emptying, reduced oxidative stress, enhanced Kit expression, and reversed delayed gastric emptying.

The density of ICC in the distal stomach was also reduced in streptozotocin-induced diabetes in rats, and loss of connectivity between nerves and ICC-IM was observed in the fundus (395). These changes were associated with decreased gastric emptying in diabetic animals. Others have reported similar finding for streptozotocin-induced diabetes in rats and found significant loss of ICC in the antrum (267). These authors also found an association between retention of ICC and upregulation of HO1 expression. For example, HO1 expression was enhanced in the corpus and ICC networks were essentially unchanged in that region. Antral ICC were depressed, and this was associated with no enhancement in expression of HO1. Loss of ICC was reversed by induction of HO1 expression by cobalt protoporphyrin. The reasons for loss of ICC in NOD/LtJ mice have been investigated. Reduced IGF-I and insulin signaling, and not hyperglycemia, were found to be responsible for the loss of ICC in diabetes (162). Expression of insulin and IGF-I receptors were localized to enteric neurons and smooth muscle cells, and transcripts for these receptors were not resolved in ICC purified by FACS (161). The same cells that expressed IGF-I and insulin receptors also expressed stem cell factor. Loss of stem cell factor was found to be the cause of ICC loss in diabetic tissues. Therefore, signaling via IGF-I and insulin does not occur directly through ICC, but ICC maintenance depends on these growth factor receptors, as they are important in maintaining expression of stem cell factor in cells adjacent to ICC.

Type II diabetes is by far the most prominent form in human patients. C57BL/KsJ-db/db mice, a monogenic model of type II diabetes, were examined for gastric emptying and damage to ICC networks (421). c-Kit+ cell density and expression of stem cell factor were also evaluated. Compared with db/+ mice, db/db had delayed gastric emptying as well as prolonged gut transit times, and contractile activity was irregular in frequency. Cells with c-Kit immunoreactivity were reduced in antrum, small intestine, and colon, as was expression of stem cell factor. These authors concluded that loss of ICC contributes to the motility defects in type II diabetes.

Reduced ICC numbers have also been reported in samples of antral muscles from full thickness gastric biopsies from human patients symptomatic of diabetic and nondiabetic gastroparesis (105, 132, 133, 184). An interesting finding in human samples was a shift in splice variants of ANO1 in ICC from patients with gastroparesis (252). A novel splice variant that lacked exons 1 and 2 and a portion of exon 3 of ANO1 was observed in patients gastroparesis. ANO1 encodes the ion channel responsible for pacemaker currents in ICC (see sect. II D), and a shift in splice variant expression might affect the kinetics or frequency of slow waves. Expression of the unique splice variant from diabetic tissues in HEK cells resulted in decreased conductance and slower kinetics than displayed by full-length ANO1 channels.

3. Surgical resection of the bowel

Acute inflammatory responses to surgery, such as bowel resection, have also been linked to adverse effects on ICC and their associated functions. Resection of murine small intestine caused loss of c-Kit-like immunoreactivity, loss of slow waves, attenuated neural responses, and abnormal responsiveness to carbachol (425). The loss of c-Kit immunoreactivity was shown to be due to loss of ICC via electron microscopy. The lesion in ICC and loss of function decreased as a function of distance (5 cm) above and below the point of the anastomosis. Loss of ICC was acute and partially recovered within 24 h of surgery. Recovery was more rapid when muscles were treated with an iNOS inhibitor,
l-N^6-(1-iminoethyl)lysine hydrochloride (l-NIL). Animals treated preoperatively with iNOS inhibitors experienced reduced pacemaker activity only near the site of the anastomosis, and transgenic animals lacking iNOS were protected against most of the postsurgical defects in ICC networks and pacemaker activity (424).

E. Cancer

*Kit* is a protooncogene encoding a receptor tyrosine kinase. Gain-of-function mutations in the juxtamembrane domain of *Kit* are found in 75–80% of GISTs, the most common form of human sarcoma, and the idea that GISTs derive from ICC has been considered (149, 150, 195, 272). Juxtamembrane domain mutations discovered in GISTs render c-Kit constitutively active, and expression of the mutated isoforms in cells resulted in unregulated growth (149). Immunostaining with antibodies to c-Kit has been an important advance in categorizing mesenchymal tumors, and realizing that c-Kit drives the growth of GISTs has afforded a therapeutic approach to controlling GISTs. Activating mutations in PDGFRα have also been identified in GISTs. Thus it is also possible that cells of the PDGFRα^+ cell lineage could contribute to development of GISTs. Inhibition of c-Kit or PDGFRα with tyrosine kinase inhibitors reduces progression of GISTs, but these drugs need to be maintained for indefinite periods for effectiveness. This has suggested that sustained growth of GISTs may be due to proliferation of cancer stem cells that are not dependent on signaling via c-Kit for survival. Thus gain-of-function mutations in c-Kit in the progenitor cells could yield continuous output of malignant GIST cells that is not controlled by blocking the tyrosine kinase activity of c-Kit (16). It was suggested that Kit^low^ Cd44^+^ Cd34^+^ ICC stem cells are the source of GIST cells. These are c-Kit independent stem cells that are not susceptible to therapeutic approaches designed to inhibit c-Kit.

IV. INTERSTITIAL CELLS IN THE URINARY TRACT

Smooth muscle tissues of the urinary tract generate spontaneous phasic contractions and tone. The intrinsic electrical and contractile activities appear to be generated by specialized pacemaker cells. Electrical and contractile activities are also regulated by neurotransmitters, and interstitial cells may be involved in transduction of neural inputs. Electrical activity generated by specialized cells may propagate to smooth muscle cells, control membrane excitability, and generate phasic contractile activity. Two types of specialized cells have been reported: c-Kit^+^ and c-Kit^−^ cells. Recently, PDGFRα^+^ cells have been shown to be at least part of the c-Kit^−^ interstitial cell population. In the following sections, the distributions and physiological and pathophysiological roles suggested for interstitial cells of the upper and lower urinary tract are discussed.

A. Renal Pelvis and Ureter

Pacemaker activity is generated in the most proximal regions of pyeloureteric tissues, and this initiates peristaltic contractions that spread toward the distal ureter. The cells and ionic mechanisms responsible for pacemaker activity in the upper urinary tract have received considerable study. Two populations of specialized cells were observed in the renal pelvis, and their ability to generate pacemaker activity has been investigated (232). Short, spindle-shaped cells, referred to as atypical smooth muscle cells (ASMC), are located in the proximal region of ureteropelvic junction. These cells generate spontaneous electrical rhythmicity (123, 124, 201). Another type of cell, referred to as ICC-like cells (ICC-LC), has also been described. c-Kit immunopositive cells have been reported in the upper urinary tract (74, 231, 260, 261, 286, 356), but c-Kit immunoreactivity is not consistent in all species (e.g., ICC-LC in guinea pig pyeloureteric system were not labeled by c-Kit antibodies; Ref. 201). ICC-LC are mainly distributed in more distal regions of the ureteropelvic junction. ICC-LC may be involved in generation of pyeloureteric pacemaker activity (201, 232). Both ASMC (13pF) and ICC-LC (17 pF) are smaller than SMC (20 pF) based on cell capacitance measurements (181).

ASMC and ICC-LC cells display high (10–40/min) and low (1–3/min) frequency of Ca^2+^ transients, respectively (228,
Based on the frequency of Ca^{2+} transients and spontaneous transient depolarizations (STD), the dominant pacemaker cells may be ASMC. Spontaneous Ca^{2+} transients and STDs were inhibited in ASMC when extracellular Ca^{2+} was reduced or by application of cyclopiazonic acid (CPA) and 2-APB (230). Ryanodine (up to 100 μM) slightly reduced Ca^{2+} transients but failed to block STDs in ASMC (229, 230). Reduced extracellular Na^+ also decreased STDs in ASMC, and these events were not affected by Cl^- channel blocking drugs or replacement of extracellular Cl^- (230). The data suggested that pacemaker activity in ASMC is due to openings of cation-selective channels stimulated by release of Ca^{2+} from IP_3 receptor-operated stores.

A recent report, using eYFP-αSMA transgenic mice, showed that ASMC, isolated from smooth muscle cells by the absence of an A-type K^+ current and STOCs, displayed two types of inward conductances that yielded STICs and large inward currents (LICs) (181). LICs were blocked by replacement of the external Na^+ with TEA (181). Thus LICs might arise from activation of nonselective cation channels (NSCC), and these events may be responsible for STDs in intact muscles. The importance of STICs, the involvement of a Ca^{2+}-activated Cl^- conductance (CaCC), and how STICs might link to the generation of LICs are not clear at present.

ICC-LC have also been shown to generate STICs and LICs (232, 233). The conductances responsible for STICs and LICs were not affected by Cl^- channel blockers. The currents reversed close to 0 mV, suggesting the currents were due to NSCC conductances (232). Current clamp (I = 0) experiments suggested that NSCC are responsible for STDs in ICC-LC (233), and spontaneous generation of currents may be due to Ca^{2+} release via IP_3 or ryanodine receptors. In another study, interstitial cells immunonegative for smooth muscle actin (most likely ICC-LC) displayed Xe991-sensitive, Kv7 currents, spontaneous transient outward currents due to activation of large conductance Ca^{2+}-activated K^+ channels, and niflumic acid-sensitive STICs (possibly CaCC) (181). These findings were supported by immunostaining for ANO1 and KV7.5 immunostaining. How ASMC and ICC-LC interact with smooth muscle cells to generate peristaltic contractions in the renal pelvis and pyloureteric junction has not been fully clarified.

### B. Bladder

Much has been written about interstitial cells in the urinary bladder since vimentin^+ interstitial cells in the detrusor muscle were shown to display cGMP-like immunoreactivity (353). Two general classes of interstitial cells have been described on the basis of their histological distributions: suburothelial and detrusor interstitial cells. Suburothelial interstitial cells lie beneath the urothelium and are cGMP-immunopositive (121). Vimentin immunoreactivity has been used typically to identify interstitial cells in the bladder. Vimentin^+ cells have been reported in the suburothelium of guinea pig (76, 129, 215, 302) and human bladders (189, 268, 363), and there are reports of c-Kit^+ cells in the suburothelium of the human (189, 287, 288, 344) and pig bladder (260). Some studies have noted that not all vimentin^+ cells are c-Kit^+ (75, 256). Thus at least two classes of interstitial cells may exist in the bladder. It should also be noted that mast cells are also c-Kit^+ and can be found in the suburothelium and in detrusor muscles. However, mast cells are typically rounded and of a less fusiform shape than the c-Kit^+ cells described in bladder tissues (189, 287). Muscarinic receptor type 3 (M3) is expressed specifically by suburethral interstitial cells, but the significance of these receptors in interstitial cells is unknown (129, 130).

Some investigators have referred to the interstitial cells of the urothelium as myofibroblasts. These cells express vimentin and connexin 43 and are in proximity to unmyelinated nerves, suggesting possible neural interactions (107). Vimentin^+ myofibroblasts exhibit STICs that are mediated by activation of a CaCC. ATP, acting via P2Y6 receptors, increased intracellular Ca^{2+} and activated CaCC (107). Developing strains of animals expressing reporters or finding immunoreactive proteins and antibodies directed toward extracellular epitopes will be important advances for unequivocal identification of bladder myofibroblasts in future studies.

Immunohistochemical evidence for c-Kit^+ cells in the bladder detrusor muscles has been reported in human (189, 287, 288, 344), pig (260), dog (12), guinea pig (72, 75, 256, 268, 399), rat (79), and mouse (255). Transmission electron microscopy (TEM) identified a prominent network of detrusor interstitial cells (CD34^+) in human detrusor (304). These cells are described as bipolar with slender dendritic process, abundant mitochondria, rough endoplasmic reticulum, Golgi apparatus, and intermediate filaments. The cells were found at the peripheral edges of muscle bundles and occasionally could be found within muscle bundles. They were considered to be similar to the ICC of the GI tract. However, these cells were immunonegative for CD117 (c-Kit). The number of interstitial cells has been reported to increase in overactive bladder generated in animal models of partial obstruction or in patients with overactive bladder (34, 193, 216). Bladder contractions are induced by activation of cholinergic M3 receptors (8, 55, 335), and smooth muscle cells make close contacts with and are apparently directly innervated by motor neurons (114, 115). Interstitial cells are also found in close proximity to cholinergic nerves (63, 189, 256), so these cells might have neuromodulatory functions in the bladder (128, 186). It should be noted that interstitial cells immunopositive for c-Kit are not observed consistently in the bladder. Some investigators have found few cells immunopositive for c-Kit in the detrusor and suburothelium, and the few cells that are c-Kit^+ appear to be.
mast cells in mice (207, 225, 245) and humans (303). Thus the presence and significance of c-Kit+ interstitial cells in the bladder is controversial at present.

1. Functional observations on cells identified as interstitial cells

Far less is known about the functional role of c-Kit+ interstitial cells in detrusor muscles than in GI muscles. Studies of these cells have relied on identification by morphological characteristics, which are not definitive in dispersed cell populations. Nevertheless, branched cells, thought to be interstitial cells, were compared with smooth muscle cells. The branched cells expressed large-conductance Ca2+-activated K+ channels (BK), TEA-sensitive delayed rectifier K+ channels (Kv), KCNQ channels, hyperpolarization-activated cyclic nucleotide-gated channels (HCN), L-type Ca2+ channels, and T-type Ca2+ channels (7, 79, 144, 186, 253, 254). A T-type Ca2+ channel blocker inhibited spontaneous phasic contractions in detrusor strips and decreased intracellular Ca2+ transients in detrusor interstitial cells, suggesting that T-type Ca2+ channels could be a Ca2+ entry source in generating spontaneous activity (79). Application of carbachol increased Ca2+ transients and phasic contractions through the M3-Gq/11-PLC pathway in the branched cells of detrusor muscles, suggesting these cells may respond to cholinergic neurotransmission in situ (186, 256, 382).

High concentrations of the c-Kit inhibitor imatinib abolished action potential firing and significantly decreased intracellular Ca2+ transients in interstitial cells and muscle contractions (79, 217). Thus it was suggested that a tyrosine kinase inhibitor might be a useful target for treating symptoms of bladder overactivity (34, 218). However, imatinib has effects on Ca2+ channels in smooth muscles, so the acute effects of this drug may not be through inhibition of c-Kit (140).

There have been suggestions that bladder dysfunction in diabetes and spinal cord injury could be related to changes in interstitial cell distribution (54, 187). However, it must be stated that a role of c-Kit+ cells in normal or diseased bladder tissues is far from established.

2. PDGFRα+ cells in detrusor muscles

Recently, another type of interstitial cell was identified in mouse, guinea pig, and human bladders (207, 268). These cells express PDGFRα and therefore have been referred to as PDGFRα+ cells, as in GI muscles (FIGURE 10, A–C). PDGFRα+ cells are distributed throughout the bladder wall, including the lamina propria and detrusor muscle. These cells have a spindle-shaped or stellate morphology and often possess multiple processes, possibly forming networks. PDGFRα+ cells surround and are located between smooth muscle bundles, and they are often in close proximity to intramural nerve fibers.

A mouse strain expressing eGFP in PDGFRα+ cells (135) has been used to identify PDGFRα+ cells in cell dispersions and for purification of cells by FACS (207). PDGFRα+ cells...
are highly enriched in Pdgfra and Kcnm3 (SK3 channel) transcripts in relation to the whole detrusor cell population. SK3 protein was also observed in PDGFRα+ cells by immunohistochemistry but was not resolved in smooth muscle cells. SK channel modulators, CyPPA and SKA-31, hyperpolarized PDGFRα+ cells and activated SK currents under voltage clamp (239). Similar responses were not observed in SMCs held at membrane potentials simulating physiological potentials. The single-channel conductance of SK channels in PDGFRα+ cells was 10 pS, and intracellular Ca2+ activated the channels. PDGFRα+ cells displayed STOCs at potentials positive to ~60 mV, and these events were inhibited by apamin.

Recently, the SK conductance in PDGFRα+ cells was shown to be activated by purines acting on P2Y1 receptors (240). P2Y receptors (mainly P2ry1) were highly expressed in PDGFRα+ cells. ATP also induced significant hyperpolarization responses in PDGFRα+ cells under current clamp. MRS2365, a P2Y1 agonist, mimicked the effects of ATP, and MRS2500, an antagonist, inhibited ATP-activated SK currents. ATP responses were largely abolished in PDGFRα+ cells of P2ry1−/− mice. These data demonstrate that purines activate SK currents and would tend to stabilize excitability in bladder muscles, thus providing an explanation for purinergic relaxation of detrusor muscles (37, 38, 46, 257).

C. Urethra

Urethral smooth muscles also display spontaneous electrical activity. Branched cells from rabbit urethra have been isolated and studied by voltage-clamp and Ca2+ imaging (188, 338). The branched cells were identified as interstitial cells on the basis of vimentin expression, absence of smooth muscle myosin, and presence of a discontinuous basal lamina, sparse rough endoplasmic reticulum, abundant mitochondria, and a well-developed smooth ER. Significant differences were observed in the behaviors of branched cells and smooth muscle cells. The exact nature of the interstitial cells in the urethra (i.e., whether mainly c-Kit+ or PDGFRα+) is unclear (286, 282).

Urethral smooth muscles exhibit STDs and slow wave-like events. The electrical events are due to activation of CaCC in response to release of Ca2+ from intracellular Ca2+ stores (142). Ca2+ transients occur in interstitial cells in intact urethral muscles; however, these events were of lower frequency and longer duration than Ca2+ transients in smooth muscle cells. Ca2+ transients in interstitial cells were inhibited by CPA, ryanodine, and 2-APB (141), as also found in isolated interstitial cells (see below).

Isolated urethral interstitial cells generate spontaneous electrical activity, but smooth muscle cells from the urethra are normally electrically quiescent. The spontaneous electrical activity in interstitial cells appears to be due to intracellular Ca2+ oscillations that generate pacemaker currents. CaCC were activated by Ca2+ release from IP3 and ryanodine receptors on Ca2+ stores (67, 156, 339). Mitochondria also regulate the activity of CaCC in interstitial cells, and electron transport chain blockers, rotenone and antimycin A, and protonophore uncouplers, FCCP and CCCP, inhibited intracellular Ca2+ waves and abolished STICs in these cells. The evidence for functional expression of CaCC in urethral interstitial cells is strong from studies of isolated cells, but a recent report suggested that Ano1 is expressed in smooth muscle cells, but not in interstitial cells (325). Thus determining the molecular species responsible for CaCC in urethral interstitial cells will be a goal for the future.

Urethral smooth muscle cells express L-type and T-type Ca2+ channels (41, 157), but expression of voltage-dependent Ca2+ channels in interstitial cells has not been determined. The Na+-Ca2+ exchanger type 3 (NCX3) has been suggested as an important contributor of Ca2+ influx in urethral interstitial cells. Lowering extracellular Na+ enhanced Ca2+ oscillations, and NCX inhibitors, KB-R7943 and SEA0400, decreased Ca2+ oscillations and STICs. Thus the Ca2+ influx mode of NCX may drive or regulate spontaneous Ca2+ release events and electrical rhythmicity (40). How NCX3 is switched to reverse mode in interstitial cells is not fully understood, but might occur by localized increases in intracellular Na+ due to actions of other transporters or cation channels.

Urethral interstitial cells may also have a role in transducing or modulating neural inputs from autonomic motor neurons. Adrenergic neurotransmission affects urethral tone via postjunctional α1 adrenoceptors (39). Norepinephrine (NE) activated STICs in interstitial cells, and this is blocked by α1 antagonists, a phospholipase C inhibitor, IP3 receptor antagonists, and CaCC blockers (341). NO is a prominent inhibitory neurotransmitter in the urethra (9), and inhibitory effects are mediated through activation of protein ki-
nase G (285). NO donors and membrane-permeable cGMP analogs inhibited STICs in urethral interstitial cells by reducing intracellular Ca\(^{2+}\) waves (340). NO may also target cyclic nucleotide-gated channels (CNG), which are expressed in urethral interstitial cells (381).

V. INTERSTITIAL CELLS IN THE REPRODUCTIVE TRACT

A. Uterus

The uterus has extraordinary requirements to accomplish the tasks of implantation and embryogenesis. The hypertrophy that accommodates the growth of an embryo and maintenance of a low level of smooth muscle excitability facilitate uterine function during pregnancy. Several studies have reported the presence of ICC or ICC-like interstitial cells in the human uterus (60, 89, 170, 171, 290, 345), but the role of these cells in uterine contractile activity and maintenance of pregnancy is poorly understood.

A few studies have attempted to study the role of c-Kit\(^{+}\) ICC-like cells in the uterus by testing the effects of imatinib mesylate (5, 169). This drug caused concentration-dependent (100 \(\mu\)M) inhibition of spontaneous uterine contractions and contractions initiated by elevated external K\(^{+}\). It is possible that the effects of imatinib were mediated by a tyrosine kinase independent signaling pathway since the concentrations required to affect contractions were much greater than concentrations required to inhibit c-Kit (69). When acute and sustained application of imatinib was studied in GI muscles, it was found that chronic application for several days was required to affect c-Kit\(^{+}\) populations of cells and inhibit electrical rhythmicity (23, 139). ICC networks were lost from the small intestines after at least 3 days of imatinib treatment (3–5 \(\mu\)M), and this was similar to the time course of the effects of neutralizing c-Kit antibodies on ICC and pacemaker activity. In studies of gastric antrum, imatinib (10 \(\mu\)M) was shown to reduce spontaneous contractions, intracellular Ca\(^{2+}\) transients, and basal [Ca\(^{2+}\)], levels without affecting pacemaker activity (139). Thus imatinib may generally suppress spontaneous contractions of smooth muscles by blocking L-type Ca\(^{2+}\) channels.

Studies of cells isolated from uterine muscles and identified as interstitial cells are limited. Cells distinct from myocytes, based on morphology and ultrastructure, were obtained from pregnant human and rat uterus (89, 427). The ICC-like cells were multipolar with spiderlike projections and enlarged nuclear regions, but c-Kit immunoreactivity was not resolved (89). The cells were noncontractile and contained no myofilaments, but numerous mitochondria, caveolae, and intermediate filaments were observed and the cells were immunopositive for vimentin (89). The cells had resting membrane potentials of \(-58\) mV and displayed no inward currents or action potentials in contrast to myocytes. Outward currents were elicited by depolarization of the ICC-like cells. The numbers of ICC-like cells increase in pregnancy, and the suggestion was made that the cells might help to stabilize uterine excitability during the later stages of pregnancy (89, 427).

B. Oviduct

Oviducts, or fallopian tubes in humans, are smooth muscle-lined tubular organs that transport ova from ovary to uterus (284). Several stages of the reproductive process occur within the oviduct and are subject to physiological regulation by a variety of processes. The four main functions of the oviduct include 1) transport of the ovum from the ovary to the site of fertilization. After delivery to the oviduct, oocytes are propelled down the tube, assisted by peristaltic contractions and oviductal fluid moved by ciliated epithelial cells, into the ampulla where fertilization takes place (134). 2) The oviduct aids in transport of spermatozoa from the site of deposition to the site of fertilization within the ampulla. Delivery of sperm to the site of fertilization is also thought to involve muscular movements of the oviduct (3). 3) The oviduct provides a suitable environment for the egg. Secretions along the oviduct provide a suitable environment and maintain the viability of the egg during the descent through the oviduct (71). 4) Transportation of the fertilized ovum (embryo) to the uterus where implantation and further development occurs. After fertilization, the developing morula descends through the isthmus of the oviduct relatively rapidly, followed by successful implantation within the endometrium of the uterus (70).

Video imaging and spatiotemporal mapping showed that cumulus oocyte complex (COC) movements are due to the contractile activity of the myosalpinx, and ciliary beating stirs the fluid environment that provides lubrication and viability to the egg and sperm (1, 192). Thus the source of peristaltic contractions in oviduct is important to understanding the physiology of this organ. Morphological studies using light and electron microscopy identified ICC-like cells in the fallopian tube, and it was postulated the cells might provide pacemaker activity (290, 291, 346). These studies described elongated c-Kit\(^{+}\) cells with dendritic processes located within the circular muscle layer and also between the circular and longitudinal muscle layers in all segments of the oviduct. The elongated c-Kit\(^{+}\) cells were distinct from the rounded and unbranched mast cells also detected within the muscularis. c-Kit\(^{+}\) cells were also detected beneath the epithelium in a layer that was 10–15 \(\mu\)m thick. The ICC-like cells represented \(\sim\)9 and 7% of total cell population in the lamina propria and muscularis, respectively (291). Although no direct functional evidence was presented, based on physiological studies in the GI tract, it was proposed that the ICC-like cells might provide pacemaker activity and/or mediate neurotransmission.
ICC have also been described in oviducts of mouse and hen (85, 117). In mice and monkeys, ICC, termed ICC-OVI, are found within the myosalpinx and organized into an extensive network throughout the oviduct (Figure 10, D and E). Functional studies demonstrated the importance of ICC-OVI in oviduct motility. Selective removal of ICC-OVI using a c-Kit neutralizing antibody resulted in loss of ICC, electrical slow waves, and propulsive contractions of the oviduct (85). It is not known whether removal of ICC-OVI leads to loss of egg propulsion and infertility as these studies were performed in neonatal animals where ICC-OVI phenotype is susceptible to neutralizing antibody (23). PDGFRα+ cells are also widely distributed in the oviduct (FIGURE 10A).

Mice infected with *Chlamydia muridarum* develop dilated oviducts, pyosalpinx, and loss of spontaneous contractile activity (85). Morphological inspection showed disruption of ICC-OVI networks, and electrophysiological recordings showed loss of pacemaker activity without change in basal smooth muscle membrane potential. Although spontaneous activity was absent in infected mice, membrane depolarizations, similar in amplitude and duration to slow waves, could be evoked by electrical field stimulation, suggesting the oviducts remained excitable. Chlamydia-induced infection of oviducts was associated with increased expression of inducible nitric oxide synthase (Nos2) and prostaglandin synthase 2 (*Ptgss2*) in stellate-shaped, macrophage-like cells that lie in close proximity to ICC-OVI in the oviduct. A correlation was shown to exist between damage to ICC-OVI and loss of pacemaker activity and increased Nos2 expression. It was suggested that loss of ICC-OVI could be a major contributing factor to the development of tubal factor infertility.

**C. Prostate**

Movement of prostatic contents from the peripheral acini into the prostatic ducts is driven by myogenic activity in the stromal wall (94). Interstitial cells, immunopositive for c-Kit, were identified in guinea pig prostate stroma, and these cells were in close contact with smooth muscle cells and varicose axon bundles (94). Examination of tissues with electron microscopy showed typical smooth muscle cells and interstitial cells with more electron-dense cytoplasm, ovoid nuclei, an abundance of mitochondria, rough ER, some caveolae, and an incomplete basal lamina. Thick filaments were not observed in these cells. These are general ultrastructural features of the ICC of the GI tract. Interstitial cells have also been reported in human prostate tissues (347). A slow wave-like electrical event drives phasic contractions of prostate smooth muscles, and these events may be driven by the c-Kit+ interstitial cells (270).

Prostate interstitial cells generate spontaneous Ca2+ transients that are abolished by nicardipine, suggesting that Ca2+ influx through L-type Ca2+ channels is a crucial factor for intracellular Ca2+ oscillations. However, an early study reported that the STDs were not affected by nifedipine (94) in the same species. CPA, caffeine, or reduced extracellular Ca2+ also abolished spontaneous Ca2+ transients, suggesting that intracellular Ca2+ stores are involved in the generation of Ca2+ transients in interstitial cells (226).

Interstitial cells in the prostate are associated with sympathetic neurons, and the interstitial cells express α1-adrenoceptors and the gap junction protein connexin 43 as demonstrated by immunohistochemistry (394). Norepinephrine evoked inward currents in isolated cells identified as interstitial cells, and these responses were blocked by phentolamine and prazosin, suggesting the responses to norepinephrine were mediated by α1-adrenoceptors. These authors suggested that interstitial cells may be targets for adrenergic regulation of contractions in prostatic ducts.

Interestingly, GISTs have shown a relationship with prostatic GIST, either primarily or as a result of metastasis (6, 77, 83). Thus expression of CD117 (c-Kit) might be useful for the diagnosis of GIST-like tumors in the prostate.

**VI. SUMMARY AND CONCLUSIONS**

Smooth muscle tissues contain a variety of interstitial cells. These cells provide important regulatory controls in normal and pathophysiological responses of smooth muscles, and we are just beginning to understand the full range of behaviors imposed by interstitial cells on the mechanical performance of smooth muscles. In the GI tract, several basic regulatory functions have been attributed to interstitial cells and a mechanistic understanding of these cells is developing. Electrical coupling between smooth muscle cells and interstitial cells is a key structural feature that plugs interstitial cells into smooth muscle cells, forming the SIP syncytium. ICC and PDGFRα+ cells express unique conductions and generate spontaneous transient inward and outward currents, respectively, that modulate the excitability of the SIP syncytium. ICC are pacemaker cells, generating electrical slow waves that conduct to smooth muscle cells and provide the basis for the phasic contractions of muscle strips and segmental and peristaltic contractions of GI organs. ICC and PDGFRα+ cells are innervated and contribute to the transduction of neural inputs from enteric motor neurons, and they appear to be specialized, generating responses to specific neurotransmitters. ICC have also been shown to have a role in mechanotransduction; however, the specific mechanosensitive responses of each cell type in the SIP syncytium and the contributions of each cell to integrated mechanosensitive responses of GI organs is not fully understood at present. More research is needed to understand the complete molecular apparatus required for development and maintenance of pacemaker activity in ICC. A
complete description of the ion channels and transporters responsible for generating and sustaining pacemaker activity may provide ideas for drugs that can normalize gastric dysrhythmias or improve slow colonic transit. Therapeutic benefits might also be realized by understanding the postjunctional mechanisms involved in neuro- and mechanotransduction.

It is very difficult to deduce the role of specific cell types in tissues as complicated as smooth muscles, particularly when the cells are electrically coupled. Functional studies on muscle strips and expression studies on whole muscle extracts do not reveal the cells that express specific genes or are responsible for specific functions. Having fluorescent reporters expressed in SIP cells makes it possible to isolate and purify each cellular component. Studies focused on the cellular components are building data bases about the unique molecular and functional features of each class of cell in the SIP syncytium. The integrated output and responses of the SIP syncytium are fundamental to GI motility. We suggest that the classic term myogenic to describe the subneural, subhormonal level of motor regulation in the gut is limited and not inclusive of the important behaviors and regulation provided by interstitial cells. Thus the term SIPgenic is suggested as a replacement for “myogenic,” or to be used as an intermediate term, to incorporate the integrative functions and full range of activities and responses of smooth muscle cells and interstitial cells of the SIP syncytium.

Based on the anatomical localization and morphologies of interstitial cells, there are subclasses of SIP cells. Therefore, better vital cellular markers or reporters driven by promoters of genes expressed in specific subclasses will be needed for efficient molecular and functional subclassifications of interstitial cells. Questions about why slow waves are propagated actively by ICC-MY but not by ICC-IM of the fundus, why interstitial cells develop in specific anatomical niches, and why there are differential sensitivities to inactivation of c-Kit will require a finer level of granularity in future studies of SIP cells.

Tissue engineering efforts have considered growing smooth muscle tissues and organs for reconstructive therapies. Maintenance of the smooth muscle phenotype is a continuing obstacle in these efforts, as smooth muscle cells redifferentiate readily when isolated from the native extracellular matrix. Another issue to consider in tissue engineering, and indeed in studies of cultured “smooth muscle cells,” is the impact of losing or abnormal growth or development of interstitial cells in culture. Few studies of cultured smooth muscles have evaluated the content or status of interstitial cells and either loss- or gain-of-function or loss of growth factor support might impact the behavior of smooth muscle cells in culture or in cultured muscle tissues. Expression studies that include cellular markers for the many cell phenotypes at play in smooth muscle tissues should be included in studies of cultured smooth muscles and tissue engineering protocols to better understand the fates of cell types that are important to the performance of native muscles.

A better understanding of the roles of interstitial cells in non-GI muscles is necessary. Studies of interstitial cells in many smooth muscle organs have stalled somewhat after the initial morphological descriptions of these cells. It is tempting to speculate that similar cells will be widespread in smooth muscle tissues and have important functions in regulating smooth muscle excitability or in mediation of responses to neurotransmitters. However, the lack of phenotypes in various smooth muscle tissues of animals with Kit mutations suggests that c-Kit+ cells may not be present or important in some smooth muscle organs. For example, the presence and functional significance of c-Kit+ interstitial cells, other than mast cells, in bladder detrusor muscles, is questionable. Use of imatinib mesylate is a poor method of testing the function of interstitial cells because the concentrations needed for acute effects have nontarget effects on voltage-dependent Ca2+ channels and/or the contractile mechanism of smooth muscle cells. These effects are downstream from generation of pacemaker activity and/or transduction of neural responses by interstitial cells. Effects of tyrosine kinase inhibitors on interstitial cells are likely to require long-term treatments to block the signaling functions of c-Kit or PDGFRa. New studies examining the presence of PDGFRa+ cells should be performed in various types of smooth muscles. Finding these cells in the bladder has provided novel hypotheses about the role of interstitial cells in this organ. Strains of mice with reporters expressed in interstitial cells should be used in studies of non-GI muscles. These experiments will give the opportunity to make unequivocal identification of cells purported to be interstitial cells and provide better cell preparations for determination of interstitial cell functions.

Considerable additional work is needed to understand the development and plasticity of interstitial cells. Many studies have made associations between failures of interstitial cells to develop or loss of cells in adults and motor dysfunction, but the factor(s) responsible for developmental failures or interstitial cell loss have not been identified. One intriguing lead is that many of the pathological conditions that result in loss of ICC are associated with inflammatory responses. Thus it is possible that these cells are victims of inflammatory mediators. At present, there is little information about the response of interstitial cells to paracrine substances, hormones, and/or cytokines. It is possible that responses or loss of responses to one of these factors during the development of pathological conditions could negatively impact interstitial cells, causing cell death or remodeling of the phenotype. Now that it is possible to purify each cellular component of the SIP syncytium, studies can be designed to understand cellular mechanisms and cellular responses to pathological challenges. Deep sequencing of tran-
scriptomes of each type of SIP cell will yield important insights into the molecular “personalities” of these cells and predict the stimuli to which the cells will respond. Having cell-specific iCRE strains will make it possible to test these hypotheses without total reliance on pharmacology. Fate mapping studies using molecular markers will make it possible to finally determine what happens to interstitial cells in response to pathological challenges. If remodeling of the interstitial cell phenotype is responsible for the disappearance of interstitial cells in pathophysiological states, then it may be possible to learn the signaling that controls remodeling and loss of interstitial cell functions and reestablishment of interstitial cells after pathological conditions are resolved. Detailed knowledge of SIP cell transcriptomes may help identify biomarkers that will provide noninvasive tests to assess the health of specific types of SIP cells in disease. As in most areas of biomedical research, molecular technologies have made rapid progress on the physiology and pathophysiology of interstitial cells possible. New dimensions of understanding of the significance of interstitial cells in the behavior of smooth muscle organs will result from these endeavors.

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Address for reprint requests and other correspondence: K. Sanders, Dept. of Physiology and Cell Biology, Univ. of Nevada School of Medicine, Reno, NV 89557 (e-mail: ksanders@medicine.nevada.edu).

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DISCLOSURES

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