Cajal beyond the gut : interstitial cells in the urinary system – towards general regulatory mechanisms of smooth muscle contractility ?

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Abstract

Interstitial cells of Cajal (ICC), similar to GI pacemakers have been identified throughout the urinary system. Although each part of the system serves a different function, ranging from peristalsis of the ureters, storage of urine by the bladder, and a sphincteric action by the urethra, they share a common mechanism in being able to generate phasic myogenic contractions. Even the urethra, often considered to be a 'tonic' smooth muscle, achieves an apparently sustained contraction by averaging numerous small asynchronous 'phasic' contractions. This activity can occur in the absence of any neural input, implying the presence of an intrinsic pacemaker. Intracellular microelectrode recordings from urethral muscle strips reveal electrical slow waves similar to those of the GI tract. To study this further, we isolated single cells from rabbit urethra and found not only smooth muscle cells (SMC), but a second cell type comprising ~10% of the total. The latter cells were branched and non-contractile and closely resembled intestinal ICC. Electrophyiological studies revealed that, while the isolated smooth muscle cells were electrically quiescent, the 'ICC' fired electrical slow waves similar to those observed in the whole tissue. The basis of this difference was the presence of a large pacemaker current involving the activation of calcium-activated Cl^- channels by spontaneous intracellular Ca^{2+} waves. These, in turn, have been shown to be modulated by neurotransmitters such as nitric oxide, noradrenaline and ATP, thus providing a possible mechanism whereby neural regulation of the urethra, as well as spontaneous tone, may be mediated via ICC. (Acta gastroenterol. belg., **2011**, 74, **536-542**).

Introduction

Interstitial cells of Cajal (ICC) have been best studied in the GI tract, where they have not only been established as important pacemaker cells, but also as stretch receptors and as intermediaries acting between nerve and smooth muscle cells in both cholinergic and nitrergic neurotransmission (1). The first suggestion that similar cells also exist in the urinary tract (UT), or indeed outside the GI tract, was made by Smet *et al.* in 1996 (2). These authors described specialised cells in guinea pig and human bladder and urethra that bore a striking resemblance to the interstitial cells of Cajal in the gastrointestinal tract. The cells they described had long processes, contained vimentin intermediate filaments and demonstrated an intense induction of cGMP immunoreactivity in response to sodium nitroprusside. Since then, other studies have described cells with a similar appearance in other locations throughout the urinary tract, including the renal pelvis and proximal ureter, the bladder and urethra (3-7). The distributions, characteristics, behaviour and possible functions of these cells in health and disease have been comprehensively reviewed elsewhere (8), therefore here we will provide only the briefest of overviews, before directing our attention to the cellular physiology of urethral ICC and their possible role in generating urethral tone.

The first question one must ask is what constitutes an ICC ? To rigorously identify a cell as such it should, strictly speaking, meet highly specific ultrastructural criteria, including possession of many mitochondria, smooth and rough endoplasmic reticulum, caveolae, a basal lamina which may be incomplete, intermediate filaments (10 nm) and absence of thick filaments and dense bodies. However, it is not always possible to apply such rigorous criteria when examining the distribution or function of these cells. The finding that GI ICC are immunopositive for the protooncogene product Kit, a receptor tyrosine kinase, has led to the extensive use of this marker to identify ICC, not only in the gut, but throughout the UT (8-10). A word of caution is necessary here though, for Kit is also a marker for other cells including mast cells, melanocytes, nerve cells and glial cells (11). Moreover, Kit staining does not always mark "ICC-like" cells in the UT and, even in the GI tract, ICC show a variable dependency on Kit signalling for their function (3,7,12).

Although ICC have been more extensively studied in the UT than in any other tissue or region outside the GI tract, progress regarding their function has lagged behind GI ICC. A major problem is that no appropriate animal model comparable to the Kit mutant W/W^v mouse, so valuable for study of ICC function in the GI tract, has been developed for the UT. Indeed the W/W^v mutant has disappointingly failed to provide any useful information regarding the role of ICC in the urinary system (13). Another problem is that, because of the less regular structure of UT versus GI tract smooth muscle tissue, it has so far not been possible to emulate the elegant dual intracellular microelectrode recordings that helped to establish beyond reasonable doubt that ICC were the GI

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pacemakers (14). The functions that have been proposed for UT ICC unfortunately amount largely to speculation in the face of experimental evidence that has proved at best circumstantial. Thus many of the roles attributed to GI ICC have also been suggested to apply to UT ICC, including acting as pacemakers in the urethra (7), electrical conduits in the upper UT (3), stretch receptors or conduits relaying sensory information in the lamina propria of the bladder (5,15), while detrusor ICC have been proposed to be pacemakers, secretory cells or intermediaries in neurotransmission (4,16).

Does a 'tonic' muscle like the urethra require a pacemaker ?

Although each part of the urinary system serves a different function, ranging from peristalsis of the ureters, storage and expulsion of urine by the bladder, and a sphincteric action of the urethra, these tissues share a common mechanism in being able to generate phasic myogenic contractions. Even the urethra, often considered to be a 'tonic' smooth muscle, achieves an apparently sustained contraction by averaging numerous small asynchronous 'phasic' contractions in a manner that has been compared to the asynchronous recruitment of motor units in skeletal muscle (7). This is well illustrated by examining the intracellular Ca^{2+} within a small area of rabbit urethral smooth muscle, measuring approximately 100 × 100 μm, loaded with fluo-4 am (Fig. 1). Ca^{2+} fluctuations in 3 regions of interest (ROI) i, ii & iii are shown. Under unstimulated conditions, each of these small ROI exhibited spontaneous Ca^{2+} oscillations, with little or no synchronicity between them. Electrical field stimulation (EFS) was then applied at a frequency of 4 Hz to stimulate the excitatory nerves. This resulted in an increase in the frequency of phasic Ca^{2+} oscillations in each of the ROI, with little evidence of tonic increase in Ca^{2+}. The summated effect of all of the events within the larger 100 × 100 μm area (defined in Fig. 1 by red box) was assessed by examining the average fluo-4 fluorescence over the whole area. In this case, oscillatory activity in the control period was less evident and there was now an apparent tonic increase in Ca^{2+} during stimulation, albeit with some superimposed phasic activity. This example illustrates that, even at a microscopic level, averaging of phasic activity can already be seen to begin to produce an overall tonic effect. It is not difficult to imagine how similar activity from myriads of small muscle bundles in the whole urethra can produce smooth changes in tone. These results suggest that there are multiple pacemakers within the urethra, but unlike in the gastrointestinal tract, these are not well networked.

Recordings with intracellular microelectrodes have also confirmed the essentially phasic nature of the urethra, with the appearance of either regular 'slow wave' depolarisations, similar to those of the gastrointestinal tract (17,18), or regular depolarisations with superimposed Ca^{2+} spikes (19). The work of Hashitani and

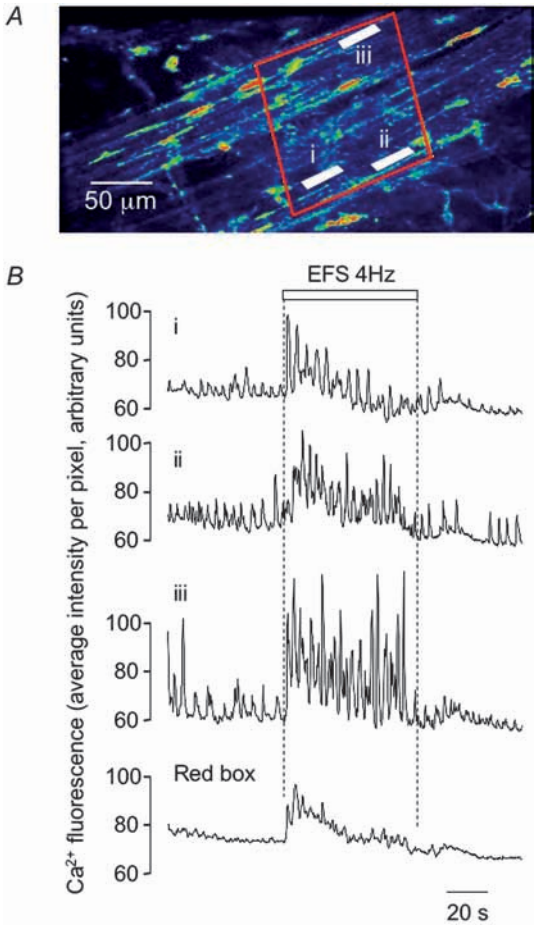

Fig. 1. — Tonic activity in the urethra is a result of spatially averaged phasic activity. *A)* A small strip of rabbit urethral smooth muscle was loaded with fluo-4AM to record changes in intracellular Ca^{2+} concentration. Three small regions of interest (ROI) were defined (i-iii) within an area approximately 100 × 100 μm (defined by red box). *B)* Ca^{2+} fluctuations in each ROI (i-iii) are shown. Note that phasic activity was present at rest within each region, with no obvious co-ordination of activity between regions. Electrical field stimulation (EFS) was then applied to stimulate autonomic nerves, causing the phasic activity in each ROI (i-iii) to increase in amplitude. In contrast, phasic activity was less evident in the larger region (red box) than in the individual ROIs, both during the control period and during EFS.

colleagues (17,18) strongly suggested that the driver for these depolarisations was Ca^{2+}-activated Cl^- current, switched on by release of Ca^{2+} from intracellular stores. The first indication that this activity might be generated by ICC, rather than by the smooth muscle cells (SMC) themselves, came when both cell types were isolated from the rabbit urethra and their electrical characteristics studied with the patch clamp method (7). It was found that SMC were electrically quiescent, although they could respond to depolarizing stimuli by firing action potentials. In contrast, the ICC fired electrical 'slow

waves' that bore a remarkable similarity to those recorded in intact urethra by Hashitani (17). The similarity of the two situations was further emphasized when it was found that ICC expressed a large Ca^{2+}-activated Cl^- current, while this current was virtually absent in the SMC (7). The discovery that urethral ICC fire spontaneous Cl^- currents is one of the rare instances in which urinary tract ICC research actually pre-empted that in the GI tract, where the pacemaker current has only recently been shown to be mediated by ANO1 Ca^{2+}-activated Cl^- channels (20).

The Pacemaker Mechanism in Urethral ICC.

The spontaneous Cl^- currents in urethral ICC were soon shown to depend on Ca^{2+} release from the endoplasmic reticulum (ER) Ca^{2+} store (21). When held under voltage clamp, ICC fired both 'spontaneous transient inward currents' (STICs), due to activation of the Cl^- current, but also 'spontaneous transient outward currents' (STOCs), due to activation of large conductance Ca^{2+}-activated K^+ channels (BK channels). As Ca^{2+} can be released from the ER store by opening either of two different Ca^{2+} channels located on the ER membrane, namely inositol trisphosphate receptors (IP_3R) and ryanodine receptors (RyR), it was of interest to investigate which of these was involved in the activation of STICs and STOCs. Intriguingly, the STICs were selectively blocked by 2-aminoethoxydiphenyl borate (2-APB), an inhibitor of release from IP_3R, while the STOCs, especially those of short duration, were resistant to this compound (21). In contrast, *both* STICs and STOCs were blocked by ryanodine, an antagonist of RyR. A clue to understanding these observations is to realize that there is a 100-fold discrepancy in ionic conductance between the rather small Cl^- channels, typically 3 pS (22) and the large BK channels (~300 pS). Thus, activation of a relatively small number BK channels by a localised but intense Ca^{2+} signal (a Ca^{2+} 'spark') could account for a significant BK current, but would activate too few Cl^- channels to produce significant current. Conversely, a widely distributed Ca^{2+} signal could activate a large number of Cl^- channels, producing a large Cl^- current, but might not be intense enough to activate the BK channels, which are less Ca^{2+}-responsive, particularly at hyperpolarised membrane potentials. This suggests that the Cl^- and BK channels are activated by different intracellular Ca^{2+} signals in urethral ICC.

The most striking feature of urethral ICC is revealed by loading them with fluo-4 am and studying intracellular Ca^{2+}, using fast scanning laser confocal microscopy (23). Under these conditions the vast majority of cells were found to fire spontaneous propagating Ca^{2+} waves that could travel the full length of the cell. 2-APB prevented the propagation of the Ca^{2+} waves, but left behind multiple localised Ca^{2+} release events, while ryanodine completely eliminated all of the Ca^{2+} events. Our interpretation of these results was that a Ca^{2+} wave was initiated by a localized release of Ca^{2+} from RyR (Ca^{2+} 'spark'), but was propagated by regenerative release from IP_3R, involving Ca^{2+}-induced Ca^{2+} release from these channels. This would also explain the observation that 2-APB selectively blocked STICs, while ryanodine blocked both STICs and STOCs.

As Ca^{2+} waves in smooth muscle cells usually require stimulation by an exogenous IP_3-generating agonist (24), an important question remaining in ICC is how they can generate spontaneous Ca^{2+} waves in the absence of such stimulation. Perhaps there is high constitutive production of IP_3 in ICC, or the IP_3R in ICC are more inherently Ca^{2+}-sensitive than those of SMC. Alternatively, other factors such the arrangement of intracellular organelles to create unique Ca^{2+} microdomains in ICC may prove to be important. In this respect the mitochondria may play a crucial role. It has been shown in a variety of cell types that the temporal and spatial profile of intracellular Ca^{2+} signals are regulated by the Ca^{2+} handling properties of the mitochondria, independently of their ability to synthesize ATP (25). In the gastrointestinal system, several studies have suggested that they play a crucial role in pacemaking (26,27). This was followed by two studies in rabbit urethral ICC, where the effect of interfering with mitochondrial Ca^{2+} uptake was examined on the cytosolic Ca^{2+} waves (28,29). The Ca^{2+} entry pathway into the mitochondria is believed to be via a specific uniporter, while the driving force for entry is created by the large negative membrane potential at the inner mitochondrial membrane, due to translocation of H^+ from the matrix to the intermembrane space. When the mitochondrial membrane potential is dissipated, for example using protonophores such as FCCP or CCCP, or with electron transport chain inhibitors, such as antimycin A or rotenone, cytosolic Ca^{2+} waves ceased. These effects were not due to ATP depletion, as inhibition of ATP synthesis with oligomycin did not affect the waves (28). Indeed, the fact that Ca^{2+} waves were abolished by 2-deoxy-glucose suggests that glycolysis, rather than oxidative phosphorylation, provides the source of ATP for spontaneous Ca^{2+} oscillations (29). The idea that the Ca^{2+} uptake by the mitochondria contributes directly to pacemaking activity is further supported by the observations that activating the uniporter with the plant flavonoid, kaempferol, increased the frequency of Ca^{2+} waves, while blocking the uniporter with RU360 abolished waves (28,29). In contrast, blocking the Ca^{2+} exit route from the mitochondria with the mitochondrial Na+/Ca^{2+} exchange blocker, CGP37157, slowed the frequency of Ca^{2+} waves (29). Although these experiments all point to a role for mitochondria in ICC pacemaker activity, the details of how they do so are unclear. It is easy to propose different models, based on the literature on other cells types. For example, there is evidence that there are close contacts between the mitochondria and ER that create Ca^{2+} microdomains that allow transfer of Ca^{2+} between the ER and mitochondria, or that mitochondria may be involved in refilling of ER (25).

Alternatively, mitochondria might be in close contact with the plasma membrane so that they influence Ca^{2+} influx by shunting Ca^{2+} from the sub-membrane space. Or perhaps mitochondria regulate Ca^{2+} concentration in the vicinity of the IP_3R, thus affecting feedback (+ve and –ve) of Ca^{2+} on Ca^{2+} release (30). However, while there is sound evidence for these ideas in other cell types, the exact role of mitochondria in ICC pacemaking remains to be elucidated.

Calcium waves in urethral ICC are not just dependent on ER Ca^{2+} release, but also require extracellular Ca^{2+}. This is well demonstrated by the fact that Ca^{2+} waves in rabbit urethral ICC rapidly ceased when external Ca^{2+} was removed (23). Although these cells possess L-type Ca^{2+} channels, these do not appear to participate to any great extent in initiating pacemaking as nifedipine had little effect on the frequency of waves, although it sometimes shortened their duration, suggesting L-type channels play a part in maintaining the plateau of the slow waves (23,31). Another potential route for Ca^{2+} influx is capacitative Ca^{2+} entry (CCE), where depletion of intracellular Ca^{2+} stores leads to activation of transmembrane Ca^{2+} influx (32). Although urethral ICC demonstrate a clear CCE mechanism, and several antagonists of this pathway were identified, none of these blockers affected spontaneous activity in these cells, suggesting that another influx pathway must predominate (33). In a subsequent study, a role for reverse mode Na^+/Ca^{2+} exchange (NCX) across the plasma membrane was proposed (34). NCX, where three Na^+ are exchanged for one Ca^{2+}, is normally thought of as a Ca^{2+} removal mechanism, but under appropriate electrochemical conditions it can work in reverse mode. Two antagonists, KB-R7943 and SEA0400, believed to selectively block reverse mode NCX, were shown to abolish Ca^{2+} oscillations and STICs in urethral ICC. Other interventions that enhance reverse mode NCX (e.g. reducing extracellular Na^+ or raising extracellular K^+) increased the frequency of Ca^{2+} oscillations, providing further support for this argument. A physiologically significant role for the pathway was also supported by the fact that SEA0400 reduced spontaneous myogenic tone in the urethra.

So far only one study has been published where attempts were made to examine Ca^{2+} signalling in ICC and SMC *in situ* in the urethra (35). Overall, it was found that activity in the urethra was poorly coupled, similar to the example shown in Fig. 1. Calcium transients in ICC were sometimes seen to be well synchronized with those in smooth muscle, though such co-ordination tended to only occur at the lowest frequencies – in the majority of instances the frequency in the smooth muscle bundles was higher than in the ICC. One explanation of this observation might be that, in a loosely coupled system such as this, other ICC out of the field of view or plane of focus were also driving the smooth muscle bundles independently of the ICC in view. Although this study provided some support for a pacemaker role for ICC, it also raised some doubts, as it was not possible to show that the ICC initiated activity in the SMC. Convincing proof of a pacemaker role for may have to wait until there is a way of selectively 'knocking out' ICC, while leaving SMC intact, as has been achieved in some parts of the GI tract using the W/W^v mouse model (12).

Effect of Neurotransmitters

Noradrenaline, acetylcholine and ATP act as excitatory neurotransmitters in the urethra, while nitric oxide (NO) and other unidentified substances act as inhibitory neurotransmitters (36,37). While there is strong evidence that ICC in the GI tract act as intermediaries between the nerve terminals and smooth muscle cells (38,39), hard functional evidence for a similar association in UT ICC is lacking. However, several immunohistochemical studies have suggested that close contacts exist between Kit +ve cells and nerve terminals, including those containing neuronal NO synthase (4,15,40). Also, Smet *et al.* (2) suggested that ICC-like cells were immunopositive for cGMP and later Garcia-Pascual *et al.* (40) found increased immunoreactivity to cGMP in both ICC and SMC following nerve stimulation in the urethras of rats and sheep. Moreover, isolated urethral ICC are known to respond to all of the neurotransmitters listed above (42-45). Isolated urethral ICC responded to exogenously applied noradrenaline by increasing frequency of slow waves, STICs (42) and Ca^{2+} oscillations (Fig. 2). These

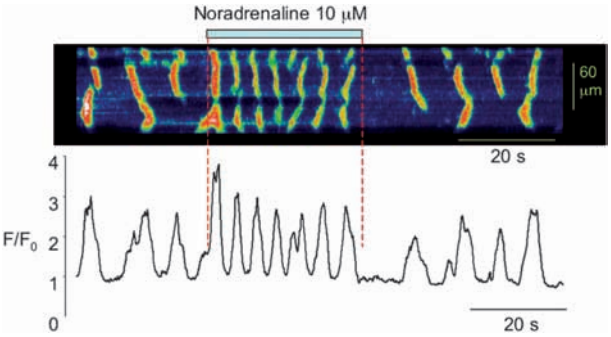

Fig. 2. — Effect of noradrenaline on Ca^{2+} waves in an ICC isolated from rabbit urethra (incubated with fluo-4AM). The image was derived from a movie by measuring fluorescence intensity at each point on a line placed along the length of the cell. The pixel intensity along a series of lines, each corresponding to a single frame of movie, were then aligned vertically left to right to construct a post hoc linescan (top panel). The vertical direction (y) corresponds to distance along the cell, the horizontal direction (x) corresponds to time, and the intensity (brightness) corresponds to the Ca^{2+} level. The intensity was pseudocoloured so that "hotter" colours correspond to increased Ca^{2+} levels. The trace (bottom panel) was derived from the linescan by measuring the average intensity at each time point. Noradrenaline increased the frequency and propagation velocity of the Ca^{2+} waves (evidenced by an increase in slope of the events).

effects are consistent with those of noradrenaline on membrane potential in whole urethra, where an increase in frequency of slow waves was observed (17). As noradrenaline is known to stimulate production of IP_3, these effects may be regarded as an up-regulation of the normal IP_3-dependent pacemaker mechanism. The fact that Cl^- channel blockers markedly reduced the contractile response to adrenergic nerve stimulation in urethral muscle strips provided some circumstantial evidence that ICC might be involved in adrenergic neurotransmission (42). Since the Cl^- current is poorly expressed in rabbit urethral smooth muscle cells, this implies a role for ICC in mediating the response, although the usual caveats, such as potential prejunctional effects of the Cl^- blockers, must be applied.

Isolated urethral ICC also responded to NO in a way that is consistent with the action of this transmitter on whole tissue, where it reduced the frequency of electrical slow waves (17). In isolated urethral ICC NO and cell permeant analogues of cGMP reduced the frequency of spontaneous depolarisations and STICs. The effect of these compounds on Ca^{2+} waves was instructive – rather than completely abolishing waves, they reduced propagation in a manner comparable to the action of 2-APB (44). This is consistent with the idea that NO mediates its effects by interfering with IP_3 signalling and is supported by a number of studies in other smooth muscle tissues showing that protein kinase G can reduce IP_3 production (46) and inhibit Ca^{2+} release from IP_3Rs (47,48). Thus, it appears that NO and noradrenaline, the two main neurotransmitters in the urethra, act on ICC by modulating the IP_3R-dependent pacemaker mechanism, inhibiting it in the case of NO and enhancing it in the case of noradrenaline.

The case for ATP appears to be a little different. We have recently shown that ATP potently increased the frequency of Ca^{2+} waves and STICs in isolated urethral ICC (45). This effect was mimicked by P2Y agonists and blocked by specific P2Y antagonists, while in isolated smooth muscle cells P2Y receptor agonists had no effect, although ATP evoked a P2X receptor cation current (37,45). Exogenously applied ATP also produced contractions in urethral muscle strips *in vitro* and, consistent with the effects on ICC, this effect was mimicked by P2Y receptor agonists and blocked by P2Y antagonists, suggesting that the effects in the tissue were mediated by ICC. While this may be true, ICC do not mediate urethral contractions evoked by purinergic nerves. In this instance, the contractions were blocked by desensitization to α,β-methylene ATP, a P2X partial agonist and by PPADS a non-selective P2 receptor antagonist, but not by selective P2Y antagonists (37). Neither α,β-methylene ATP (37) nor PPADS (Fig. 3) had any effect on ICC or their responses to ATP, while both drugs blocked the ATP-evoked cation current in smooth muscle cells. Thus, we are left with the situation where the intrinsic purinergic transmitter appears to exert its effect directly on the smooth muscle cells, while exogenous ATP, or possibly one of its metabolites, appears to act through ICC, or some other intermediary cell type.

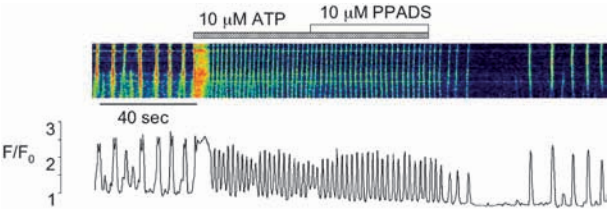

Fig. 3. — Effect of ATP and PPADS on Ca^{2+} waves in an ICC isolated from rabbit urethra. For explanation of method see Figure 2. ATP increased the frequency of the Ca^{2+} waves. This effect was not antagonised by PPADS.

Conclusion

Although the functions of ICC in any part of the urinary system have not yet been established, there is ever more information accumulating regarding their behaviour at single cell level and their distribution and association with nerve and muscle cells within the UT. The striking feature of isolated urethral ICC is their ability to generate electrical slow waves similar to those seen in intact muscle strips, while the smooth muscle cells alone do not appear to have all of the necessary mechanisms to account for this activity. This suggests that urethral myogenic tone may depend on ICC to drive electrical activity and, ultimately, contraction of the smooth muscle cells.

Prospects for Future Research

The finding that ICC are widely distributed throughout the UT poses an exciting challenge for the future in attempting to unravel their varied and perhaps even multiple functions within the different regions. Although their physiological functions are not yet clear there is increasing evidence that their density and distribution is altered in certain clinical conditions. Biers *et al.* (49) reported that Kit labelling showed significantly more ICC-like cells in human samples from overactive bladder (OAB) than in normal individuals. Since then evidence for a role in OAB has been rapidly accumulating, suggesting that ICC may provide therapeutic targets for treating this condition (reviewed in 50). Conversely in megcystis-microcolon intestinal hypoperistalsis syndrome, where the bladder is distended in the absence of obstruction, ICC are markedly reduced in number, again consistent with the idea that they play a part in initiating motor activity (51). Clearly, much work needs to be done to establish if these effects on ICC are causal or incidental in these conditions, however such findings provide strong encouragement to continue research in this field.

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