SYMPOSIUM REPORT

Ca^{2+} signalling in urethral interstitial cells of Cajal

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Interstitial cells of Cajal (ICC) in the urethra have been proposed as specialized pacemakers that are involved in the generation of urethral tone and therefore the maintenance of urinary continence. Recent studies on freshly dispersed ICC from the urethra of rabbits have demonstrated that pacemaker activity in urethra ICC is characterized by spontaneous transient depolarizations (STDs) under current clamp and spontaneous transient inward currents (STICs) under voltage clamp. When these events were simultaneously recorded with changes in intracellular Ca^{2+} (using a Nipkow spinning disk confocal microscope) they were found to be associated with global Ca^{2+} oscillations. In this short review we will consider some of these recent findings regarding the contribution of intracellular Ca^{2+} stores and Ca^{2+} influx to the generation of pacemaker activity in urethral ICC with particular emphasis on the contribution of reverse Na^{+}/Ca^{2+} exchange (NCX).

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It is now well established that ICC in specific regions of the GI tract act as pacemakers that are responsible for generating electrical slow waves which form the basis of co-ordinated waves of contraction observed throughout the gut (Thuneberg, 1982; Sanders, 1996; Dickens *et al.* 1999; Sanders *et al.* 2006). Therefore, when ICC with similar morphological and immunohistochemical properties to those in the gut were found in the smooth muscle layers of the urethra, it was suggested that they may perform a similar function (Sergeant *et al.* 2000). This idea was strengthened with the results of electrophysiological experiments which showed that urethral ICC were spontaneously active and that the pattern of electrical activity in single ICC resembled that recorded from the strips of whole tissue using intracellular microelectrodes (Hashitani *et al.* 1996; Hashitani & Edwards, 1999; Sergeant *et al.* 2006). In contrast, the majority of smooth muscle cells (SMCs) isolated from the urethra were electrically quiescent and were therefore not considered as the source of the electrical activity recorded from the whole tissue. ICC in the urethra were originally referred to as interstitial cells (IC; Sergeant *et al.* 2000). However, in order to try to standardize the

terminology in the literature they are now referred to as ICC (Sergeant *et al.* 2006; Bradley *et al.* 2006) in line with those in the gastrointestinal (GI) tract.

Pacemaker mechanism

STICs in urethral ICC were inhibited by the chloride channel blockers A-9-C and niflumic acid (Sergeant *et al.* 2000), and their reversal potential closely followed the predicted chloride equilibrium potential (E_{Cl}, Sergeant *et al.* 2000, 2006) suggesting that they were due to activation of Ca^{2+}-activated Cl^{-} channels. Although STICs with a similar pharmacological profile have been recorded from various smooth muscles, including rabbit portal vein and pulmonary artery (Wang *et al.* 1992; Hogg *et al.* 1993) and canine and guinea pig trachea (Janssen & Sims, 1994), the amplitude and temporal profile of the STICs recorded from urethra ICC were quite different. For example, STICs in isolated urethral ICC often exceeded 900 pA (Sergeant *et al.* 2001) compared to amplitudes of ~100 pA in SMCs (Large & Wang, 1996). In addition, the kinetics of STICs in ICC were much slower (> 1 s duration) in urethra ICC compared to their smooth muscle counterparts (< 100 ms duration). These differences were thought to reflect differences in the underlying Ca^{2+} signals responsible for generating STICs. Previous studies had indicated that STICs in SMCs were caused by Ca^{2+} sparks (ZhuGe *et al.* 1998; Gordienko *et al.* 1999). However, given the small single channel conductance of

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Ca^{2+}-activated Cl^- channels (2.6 pS; Klockner, 1993) it seemed unlikely that a Ca^{2+} spark would be able to activate a sufficient number of channels to generate STICs of the large amplitudes recorded in urethral ICC. Instead, it was proposed that STICs in ICC arose from global Ca^{2+} oscillations (Sergeant et al. 2001).

Role of Ca^{2+} stores

The importance of Ca^{2+} stores to pacemaker activity in urethra ICC was firmly established in a study by Sergeant et al. (2001) which demonstrated that the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitor, cyclopiazonic acid (CPA) abolished STICs. Furthermore, activation of STICs appeared to involve release of Ca^{2+} from both inositol trisphosphate

(IP_3)- and ryanodine-sensitive stores as they were abolished by the phospholipase C (PLC) inhibitor 2-nitro-4-carboxyphenyl-N,N-diphenylcarbamate (NCDC), the IP_3 receptor (IP_3R) blocker, 2-aminoethoxydiphenyl borate (2-APB) (Maruyama et al. 1997) as well as caffeine and ryanodine. The contribution of IP_3Rs and ryanodine receptors (RyRs) to pacemaker activity in urethral ICC was assessed in more detail in a later study by Johnston et al. (2005) which looked at the effects of these agents on the Ca^{2+} oscillations underlying STICs. This study showed that urethra ICC loaded with fluo-4 AM developed regularly occurring global Ca^{2+} oscillations that were associated with STICs as originally predicted by Sergeant et al. (2001). Interestingly, while application of the RyR inhibitor tetracaine completely abolished these events, inhibition of IP_3Rs with 2-APB only decreased their spatial spread and converted the propagating Ca^{2+} waves into
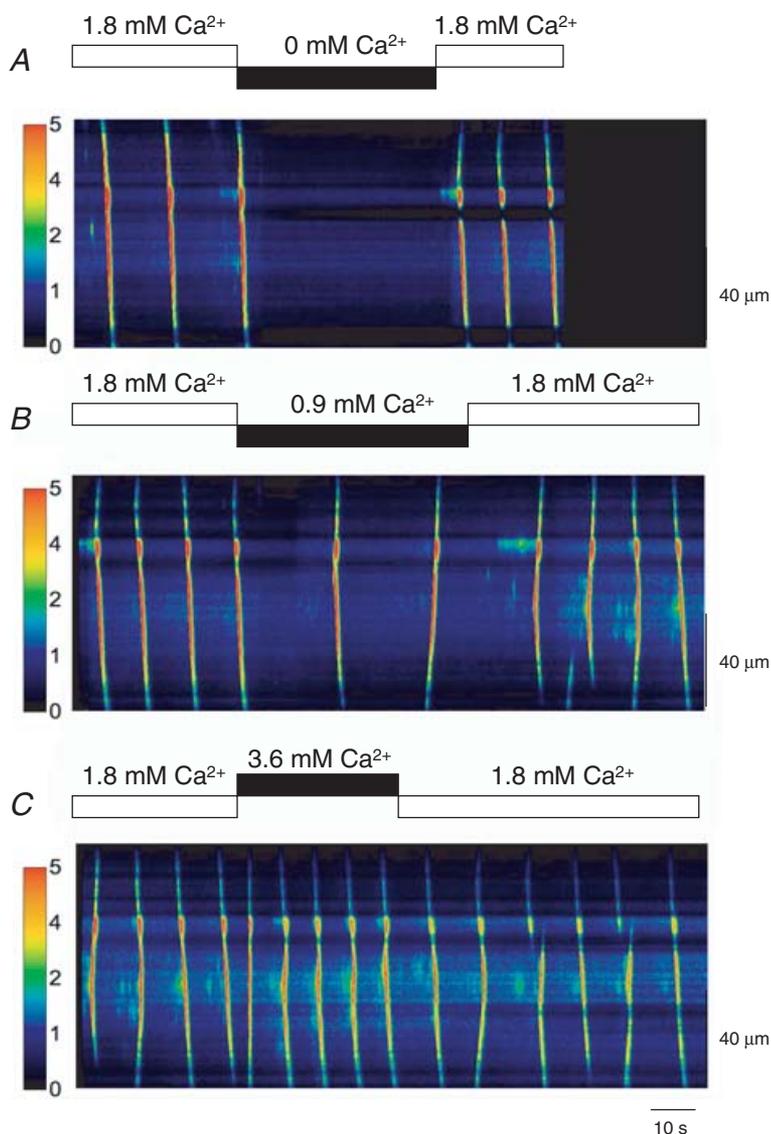

Figure 1. Pseudo linescan or *x,t* plot of propagating Ca^{2+} waves in an isolated urethra ICC
Ca^{2+} waves were abolished by removal of Ca_o^2 (*A*). Reduction in $[Ca^{2+}]_o$ from 1.8 to 0.9 mM reduced the frequency of Ca^{2+} waves by ~50% (*B*). An increase in $[Ca^{2+}]_o$ to 3.6 mM increased the frequency of Ca^{2+} waves (*C*). Modified from Sergeant et al. (2006.)
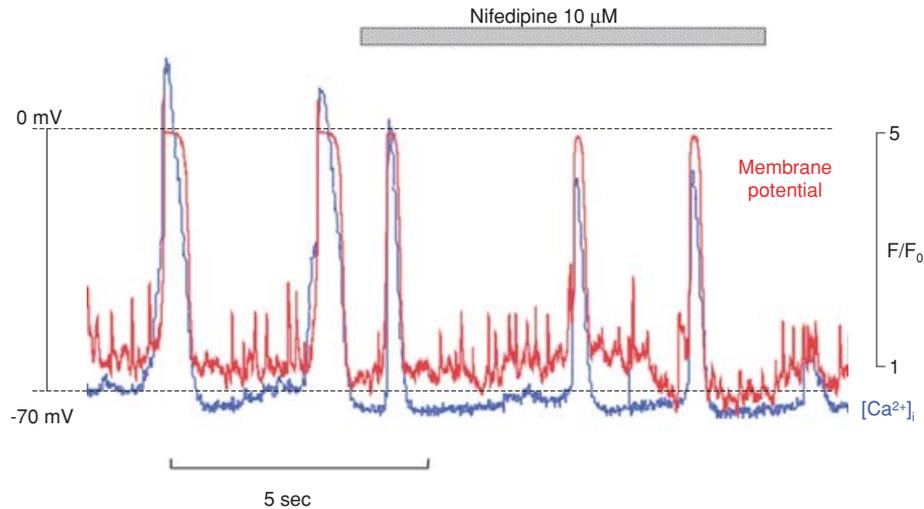
Figure 2. Simultaneous recording of membrane potential in current clamp and $[Ca^{2+}]_i$ in a fluo-4 AM loaded urethral ICC, imaged with a Nipkow spinning disk confocal microscope shows that STDs (red) are associated with spontaneous Ca^{2+} oscillations (blue)
In the presence of 10 μM nifedipine the duration of both events is shortened, but their frequency is not affected.

more localized events. Therefore, it appeared that IP_3Rs were critically involved in propagation of Ca^{2+} waves, but that RyRs were responsible for generating the primary pacemaker event. The RyRs were therefore considered the 'prime oscillators' in the pacemaker mechanism.

Ca^{2+} influx in urethra ICC

Johnston *et al.* (2005) also demonstrated that the frequency of Ca^{2+} oscillations in urethra ICC was not only dependent on Ca^{2+} release from stores but was also critically dependent on Ca^{2+} entry. For example, when $[Ca^{2+}]_o$ was elevated to 3.6 mM the frequency of the Ca^{2+} waves increased significantly. Conversely, a reduction of $[Ca^{2+}]_o$ from 1.8 mM to 0.9 mM decreased wave frequency by ∼40% and removal of $[Ca^{2+}]_o$ led to the immediate cessation of oscillations. An example of these effects is shown in Fig. 1. One possible interpretation of these results is that the reduction in $[Ca^{2+}]_o$ decreased the Ca^{2+} content of intracellular stores sufficiently to reduce wave frequency. However, Johnston *et al.* (2005) suggested that this was not the case. They showed that application of 10 mM caffeine for 10 s to a spontaneously active ICC evoked a large Ca^{2+} transient. When repeated in the presence Ca^{2+} free medium the amplitude of the caffeine response was unaffected despite the fact that spontaneous Ca^{2+} oscillations had ceased. This suggested that during the exposure to Ca^{2+} free medium (60 s) the intracellular Ca^{2+} stores remained intact.

This study also showed that the frequency of Ca^{2+} waves was inhibited by high concentrations (1 mM) of the non-specific Ca^{2+} entry blockers Cd^{2+} and La^{3+} but little affected by nifedipine (10 μM). More recent experiments

have examined the effect of nifedipine in more detail. Figure 2 shows a simultaneous recording of membrane potential in current clamp and $[Ca^{2+}]_i$ in a fluo-4 AM loaded urethral ICC, imaged with a Nipkow spinning disk confocal microscope. Under control conditions the cell exhibited regularly occurring STDs (shown in red) of ∼70 mV amplitude comprising a spike and plateau at 0 mV. These events were associated with spontaneous Ca^{2+} oscillations (shown in blue). Application of nifedipine (10 μM) slightly decreased the amplitude and duration of STDs and Ca^{2+} oscillations, but their frequency was not affected suggesting that L-type Ca^{2+} channels are not involved in the generation of pacemaker activity.

Given the importance of Ca^{2+} stores to the generation of pacemaker activity, studies were also completed to assess the contribution of capacitative Ca^{2+} entry (CCE) to the influx pathway. Bradley *et al.* (2005) showed that isolated ICC exhibited a rise in $[Ca^{2+}]_i$ in response to addition of
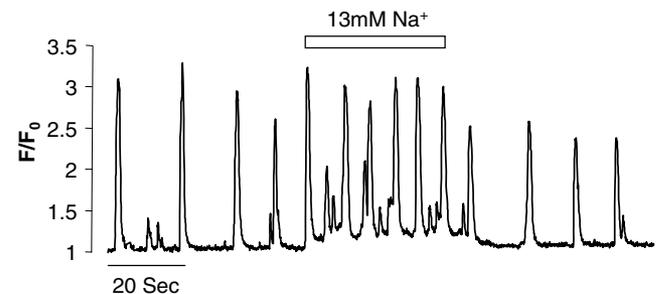

Figure 3. Representative recording from an isolated ICC showing that reduction of $[Na^+]_o$ from 130 to 13 mM doubles the frequency of spontaneous Ca^{2+} oscillations
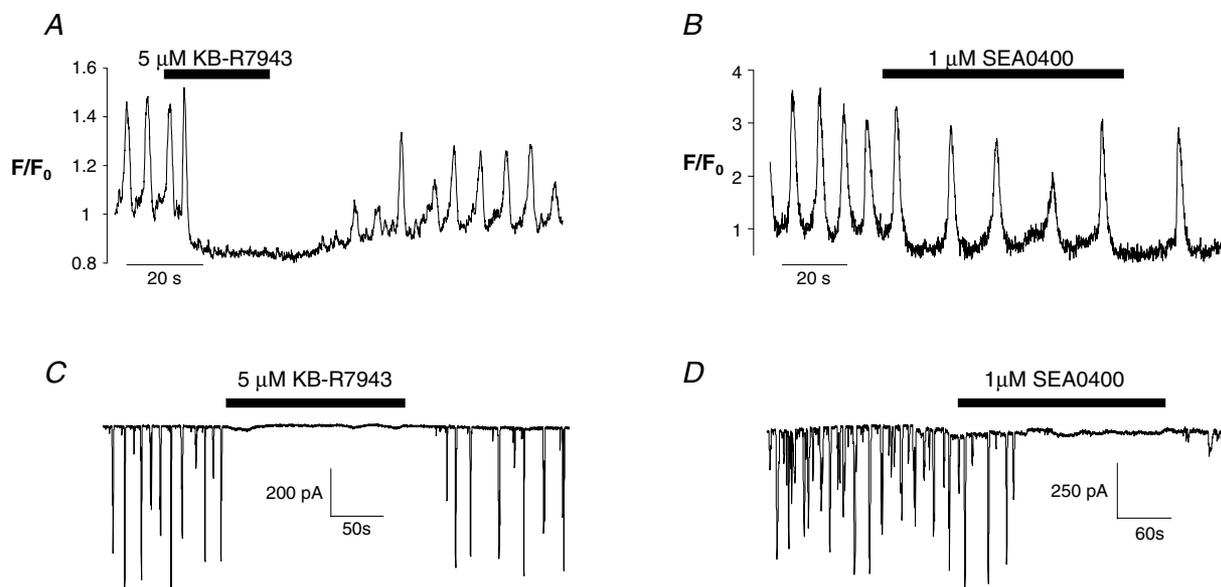Modified from Bradley *et al.* (2006).

Figure 4. Representative recordings showing the effect of the selective reverse mode NCX inhibitors KB-R7943 and SEA0400 on spontaneous Ca^{2+} oscillations (*A* and *B*, respectively) and STICs recorded at -60 mV (*C* and *D*, respectively)
Modified from Bradley *et al.* (2006).

Ca^{2+} to the bath which was much larger when Ca^{2+} stores were depleted with CPA than under control conditions. This 'overshoot' in Ca^{2+} is a hallmark of CCE in a range of cell types (Hallam *et al.* 1989; Holda *et al.* 1998; Wilson

et al. 2002) indicating that urethral ICC possessed a similar CCE pathway. CCE in urethral ICC was inhibited by Ni^{2+} (100 μM), La^{3+} (10 μM) and Gd^{3+} (10 μM), but was not affected by SKF96365 (10 μM), nifedipine (10 μM) or
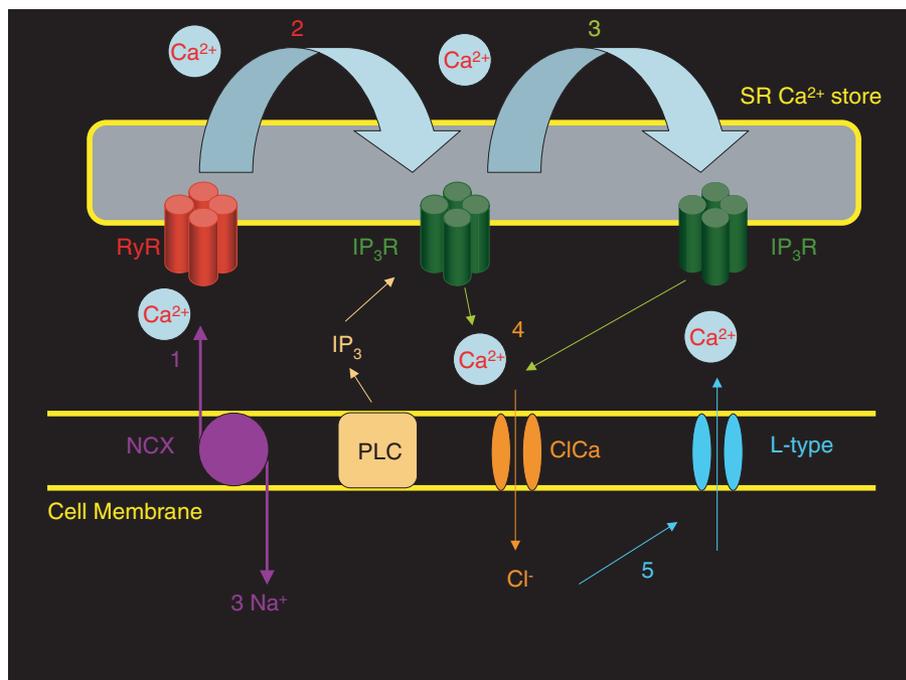

Figure 5. Schematic model of pacemaker activity in isolated urethra ICC
Ca^{2+} release from RyRs (red) is initiated by Ca^{2+} influx via reverse NCX. This in turn causes further release of Ca^{2+} from IP$_3$Rs (green) which are therefore responsible for propagation of these events. The raised intracellular $[Ca^{2+}]$ leads to stimulation of plasmalemmal Ca^{2+}-activated Cl^- channels causing depolarization of the membrane and activation of L-type Ca^{2+} channels.

wortmanin (1 μM), and only slightly diminished (21%) by 2-APB (100 μM). Interestingly, the agents which inhibited CCE did not abolish STICs, suggesting that another Ca^{2+} entry pathway must be involved in the generation of these events.

A clue to the identity of a Ca^{2+} entry pathway involved came form an observation by Putney *et al.* (2001) who noted that in cells which don't display CCE, refilling of Ca^{2+} stores is most likely achieved by Ca^{2+} influx via reverse NCX. NCX is also known to be inhibited by high concentrations of La^{3+} and Cd^{2+} (Blaustein & Lederer, 1999) which had been previously shown to inhibit Ca^{2+} oscillations in urethra ICC (Johnston *et al.* 2005). This prompted us to investigate if Ca^{2+} influx via reverse NCX contributed to pacemaker activity in isolated urethral ICC. Although NCX is typically thought of as a Ca^{2+} extrusion mechanism it is in fact a bidirectional ion transport protein which can mediate Ca^{2+} entry depending on the net electrochemical driving force acting on it (Blaustein & Lederer, 1999). Bradley *et al.* (2006) tested if interventions designed to enhance reverse mode NCX increased the frequency of pacemaker activity. They found that reduction of $[Na^+]_o$ from 130 to 13 mM (which will make E_{NCX} more negative and therefore increase the driving force for Ca^{2+} entry via reverse NCX) dramatically increased the frequency of Ca^{2+} oscillations and STICs. A representative example of the effect of 13 mM $[Na^+]_o$ on spontaneous Ca^{2+} oscillations is shown in Fig. 3. Bradley *et al.* (2006) also reported that two putative reverse NCX inhibitors (KB-R7943 and SEA0400; Iwamoto *et al.* 1996; Watano *et al.* 1996; Matsuda *et al.* 2001; Lee *et al.* 2004) inhibited or else greatly reduced the frequency of both STICs at −60 mV and spontaneous Ca^{2+} oscillations. The latter effect was consistently accompanied by a fall in basal Ca^{2+} levels suggesting that Ca^{2+} influx via this pathway was involved in setting basal Ca^{2+} levels in these cells. Representative examples of these effects are shown in Fig. 4. These effects were noted to be similar to those caused by tetracaine, but not by 2-APB (Johnston *et al.* 2005).

Wu & Fry (2001) demonstrated that caffeine-induced Ca^{2+} release could be enhanced by Ca^{2+} influx via reverse NCX in guinea pig detrusor smooth muscle cells. However, Bradley *et al.* (2006) found that KB-R7943 and SEA0400 did not significantly affect Ca^{2+}-activated Cl^- currents (I_{ClCa}) evoked by application of caffeine or noradrenaline, suggesting that reverse NCX did not affect refilling of intracellular Ca^{2+} stores in urethra ICC. These data also demonstrated that KB-R7943 and SEA0400 did not produce their inhibitory effects via non-specific actions on Ca^{2+}-activated Cl^- channels, IP_3Rs or RyRs. The effects of KB-R7943 and SEA0400 were notably similar to those produced by tetracaine (Johnston *et al.* 2005). However, as KB-R7943 and SEA0400 were shown not to inhibit RyRs directly, it suggested that Ca^{2+} influx via reverse NCX triggered release of Ca^{2+} from RyRs. Such a mechanism was reminiscent of that described by Leblanc & Hume (1990) in cardiac myocytes. In these cells reverse mode NCX was facilitated by raised $[Na^+]_i$ due to influx via a TTX sensitive Na^+ channel. Thus far we have no evidence that such a pathway exists in urethral ICC; however, any mechanism that would raise $[Na^+]_i$ in the vicinity of the exchanger would tend to favour reverse mode NCX by making E_{NCX} more negative.

Model of pacemaker activity

Based on the currently available data one cycle of pacemaker activity can be considered to comprise the following sequence of events: (1) Ca^{2+} influx via reverse NCX, (2) activation of RyRs, causing localized Ca^{2+} release events, (3) propagation of these events by opening of IP_3Rs, (4) stimulation of plasmalemmal Ca^{2+}-activated Cl^- channels causing depolarization, and (5) activation of L-type Ca^{2+} channels. This model is illustrated in the schematic diagram shown in Fig. 5.

Summary

ICC in the urethra have been proposed as pacemaker cells which may regulate spontaneous myogenic tone. In this short review we have attempted to assess some of the recent studies which have described different components which contribute to the pacemaker mechanism in isolated ICC. Studies in this field are at an early stage in comparison with those in the GI tract, but a clearer picture of the mechanisms involved is beginning to emerge and points to differences in the cellular basis of pacemaking in ICC in both regions. Further studies will be required to test the model of pacemaking proposed above, as well as to investigate the function of these cells in intact syncytia.

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