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Interstitial Cells of Cajal Review Series
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Interstitial cells of Cajal in the urethra

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Abstract

The smooth muscle layer of the urethra generates spontaneous myogenic tone that is thought to make a major contribution to urinary continence. The mechanisms underlying generation of tone remain unclear, however recent studies from our laboratory highlighted a role for a specialised population of pacemaker cells which we originally referred to as interstitial cells (IC) and now term ICC. Urethra ICC possess an electrical pacemaker mechanism characterised by rhythmic activation of Ca^{2+}-activated Cl^- channels leading to spontaneous transient inward currents (STICs) under voltage clamp and spontaneous transient depolarisations (STDs) under current clamp conditions. Both STICS and STDs are now known to be associated with spontaneous Ca^{2+} oscillations that result from a complex interplay between release of Ca^{2+} from intracellular stores and Ca^{2+} influx across the plasma membrane. In this review we will consider some of the precise mechanisms involved in the generation of pacemaker activity and discuss how these are modulated by excitatory and inhibitory neurotransmitters.

Keywords: urethra • smooth muscle • pacemaker • interstitial cells of Cajal • calcium oscillations

Introduction

Urethral smooth muscle generates spontaneous myogenic tone that is important for the maintenance of urinary continence. It is known that the level of tone can be modulated by excitatory and inhibitory neurotransmitters such as noradrenaline and nitric oxide, however there is still relatively little known about the mechanisms which lead to the development of tone in the absence of neural inputs. In this review we will discuss some of the recent advances in our understanding of the mechanisms involved in contraction of urethra smooth muscle and in particular on the role interstitial cells of Cajal (ICC) which have recently been found in this area and are proposed as specialised pacemakers. In previous publications leading up to this review we have referred to these cells in the urethra as interstitial cells. However in order to try and simplify the terminology in the literature we have now referred to them as ICC in line with those in the GI tract.

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There is now broad agreement that ICC located in the myenteric regions of the gastro-intestinal (GI) tract are responsible for generating electrical slow waves which form the basis of co-ordinated waves of muscle contraction observed throughout the GI tract [1–5]. Simultaneous measurement of contractility and membrane potential from guinea pig intestine showed that muscle contractions were associated with electrical slow waves and on some occasions driver potentials directly recorded from ICC [6]. Therefore, it is clear that rhythmic firing of pacemaker potentials from ICC in the GI tract gives rise to associated phasic contractions of the surrounding smooth muscle. The notion that tonic contraction of urethra smooth muscle may arise from a similar mechanism is relatively new. For many years it was assumed that urethral tone was generated via a similar mechanism to that proposed for arterial myocytes which were thought to rely on a sustained window current of steady state Ca^{2+} influx *via* L-type Ca^{2+} channels [7–8]. This view was consistent with the observations that myogenic tone in the urethra was inhibited by dihydropyridines [9] and that smooth muscle cells isolated from the pig urethra were electrically quiescent with resting membrane potentials of ~–40 mV [10]. However, intracellular recordings made from strips of rabbit urethra smooth muscle by Hashitani *et al*., [11] showed that the urethra produced slow wave like events which were more reminiscent of the electrical activity of the gut and did not fit with a model involving sustained Ca^{2+} influx due to the window current of L-type Ca^{2+} channels. The authors of this study noted the similarity between the pattern of electrical activity in the urethra and the GI tract where such activity originated in ICC. However, as ICC had yet to be discovered in any tissue outside the GI tract, the slow waves were presumed to originate in the urethral smooth muscle cells.

The first indication that the urethra possessed a distinct cell type with morphological similarities to ICC came from a report by Smet *et al.,* [12]. This study demonstrated that human bladder and urethra contained interstitial cells which were immuno-positive for vimentin, cGMP and bore a remarkable resemblance to the ICC in the digestive tract [1–2, 13]. These authors suggested that interstitial cells could be the targets for neuronally released nitric oxide and by analogy, act in similar fashion to ICC in the GI tract which are also known to serve as mediators of neurotransmission [14–16]. However, no pacemaker function was ascribed to these cells at this time.

Specialised pacemaker cells in the urethra?

The first physiological evidence that IC could act as specialized pacemakers in regions outside the GI tract was put forward by Sergeant *et al*., in 2000 [17]. Since this time ICC-like cells have been proposed as pacemakers in a range of smooth muscles including lymphatics [18], prostate [19], blood vessels [20], bladder [21] and fallopian tube [22]. Sergeant *et al*., [17] reported that enzymatic dispersal of rabbit urethra smooth muscle not only yielded spindle shaped smooth muscle cells but also a population of branched, non-contractile cells which appeared darker and thinner than the smooth muscle cells (see Fig. 1). These cells were termed interstitial cells (IC) and are now referred to as ICC and comprised ~5% of the total cell number. It was immediately obvious that urethra ICC were similar in appearance to freshly dispersed ICC from the canine proximal colon as described by Langton *et al*., [23] prompting speculation that they might serve a similar purpose in the urethra. Immunohistochemical studies showed that urethral ICC stained positive for the intermediate filament vimentin, unlike the surrounding smooth muscle cells. In contrast, smooth muscle cells were immuno-positive for smooth muscle myosin whereas ICC were not. Characterisation of ICC ultrastructure in the urethra revealed further similarities to those in the GI tract as noted by Thuneberg [1] and Rumessen and Thuneberg, [24]. Thus they had an incomplete basal lamina, abundant caveolae, a cytoplasm containing many mitochondria and abundant intermediate (10 nm) filaments (but not actin or myosin filaments), a well developed smooth endoplasmic reticulum and a rather sparse rough endoplasmic reticulum. This study also reported that urethral ICC were not c-Kit positive. However recent experiments from our laboratory demonstrate that urethra ICC stain positively for c-Kit in whole mount preparations (Sergeant, Hollywood, McHale, Thornbury & Ward, unpublished observations).

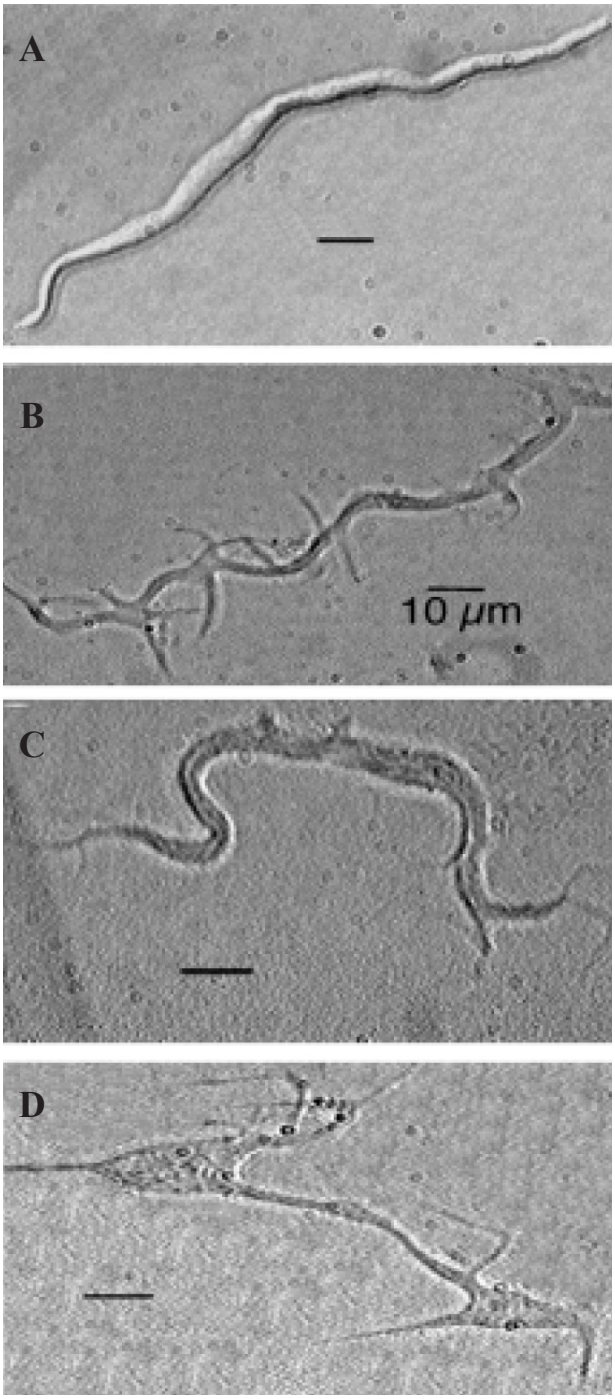

Fig. 1 Pictures of a freshly dispersed smooth muscle cells (**A**) and interstitial cells (**B**, **C** & **D**) from the rabbit urethra, shown under phase contrast. The solid line represents 10 μm scale bar in each figure. Adapted from *Sergeant et al., 2000*.

Further evidence which pointed to the possibility that the ICC reported by Sergeant *et al*., [17] could act as pacemakers came from the observation that the electrical activity of isolated ICC resembled that recorded from the whole tissue (see Fig. 2). Hashitani *et al*., [11] showed through a combination of ion substitution and pharmacological experiments that the electrical activity in intact strips of urethra smooth muscle resulted from stimulation of plasmalemmal Ca^{2+}-activated Cl^- channels due to spontaneous release of Ca^{2+} from intracellular stores. At this time the authors concluded that it was the smooth muscle cells themselves which were responsible for this behaviour. However, single cell voltage clamp experiments showed that smooth muscle cells had little or no Ca^{2+}-activated chloride currents (ICl_{Ca}) and only a small minority were spontaneously active [17]. In contrast, ICC possessed abundant ICl_{Ca} and >80% generated large spontaneous transient depolarisations (STDs), which resembled the slow wave activity described by Hashitani *et al*., [11]. Taken together these observations led Sergeant *et al*., [17] to conclude that ICC in the urethra serve as specialized pacemaker cells that regulate spontaneous myogenic tone.

Pacemaker mechanism

As ICC in the urethra resembled ICC in the GI tract it was thought that they may share a common pacemaker mechanism. Under voltage clamp conditions ICC isolated from the rabbit urethra and networks of ICC cultured from the murine small intestine develop spontaneous transient inward currents (STICs) of similar amplitude and time courses [17], [25–26]. However, the ionic basis of pacemaker activity in both tissues appears to be fundamentally different. STICs in urethral ICC were inhibited in Ca^{2+}-free medium and by the traditional chloride channel blockers A-9-C and niflumic acid [17]. Furthermore, the reversal potential of STICs closely followed the predicted chloride equilibrium potential (E_{Cl}, Fig. 3) suggesting a role for ICl_{Ca} in their generation. On the other hand the reversal potential of STICs in cultured ICC from the murine small intestine did not follow the E_{Cl} but were significantly altered by reductions in extracellular Na^+ and/or Ca^{2+} suggesting involvement of a non-selec-

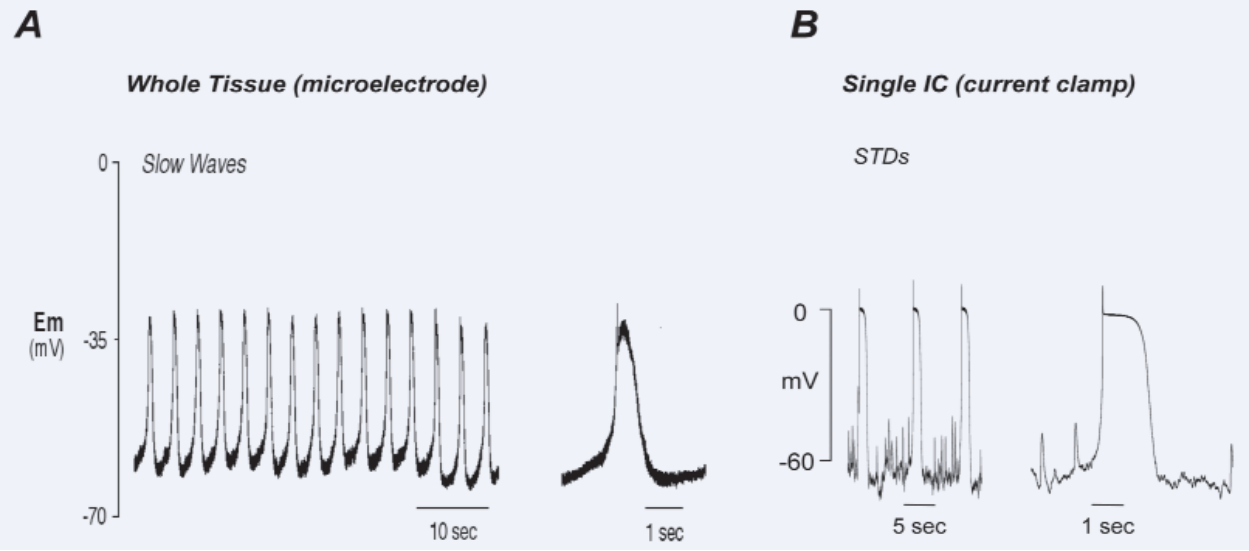

Fig. 2 Representative examples of the electrical activity recorded from intact strips of urethra smooth muscle (**A**) and single ICC (**B**).

tive cation current (I_{NSCC}) [26–27]. However, Huizinga *et al*., [28] have also reported that high conductance chloride channels may also contribute to pacemaker activity in ICC cultured from tissue explants from the murine small intestine. The exact molecular identity non selective cation channel reported by Koh *et al*., [26–27] has yet to be elucidated though it has been reported to share several characteristics with TRPc4 currents [29]. This has several important implications and points to further differences in the control mechanisms which generate the pacemaker current in both tissues, because unlike Ca^{2+}-activated Cl^- channels which are activated by rises in intracellular Ca^{2+}, TRPc4 channels open in response to reductions in Ca^{2+} at their cytoplasmic face [27, 29].

The role of intracellular Ca^{2+}-stores in the generation of STICs in urethra ICC was firmly established in a study by Sergeant *et al*., in 2001 [30]. They showed that STICs were abolished by the sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase (SERCA) inhibitor, cyclopiazonic acid (CPA). Furthermore, this study demonstrated that Ca^{2+} release from both ryanodine and inositol trisphosphate (IP_3) sensitive stores contributed to this activity since either ryanodine or 2APB [31] abolished the STICs. Although the precise nature of the Ca^{2+} events responsible for STICs was not investigated in this study, Sergeant *et al*., [30] speculated that STICs arose due to global Ca^{2+} waves and that the IP_3 sensitive store was the

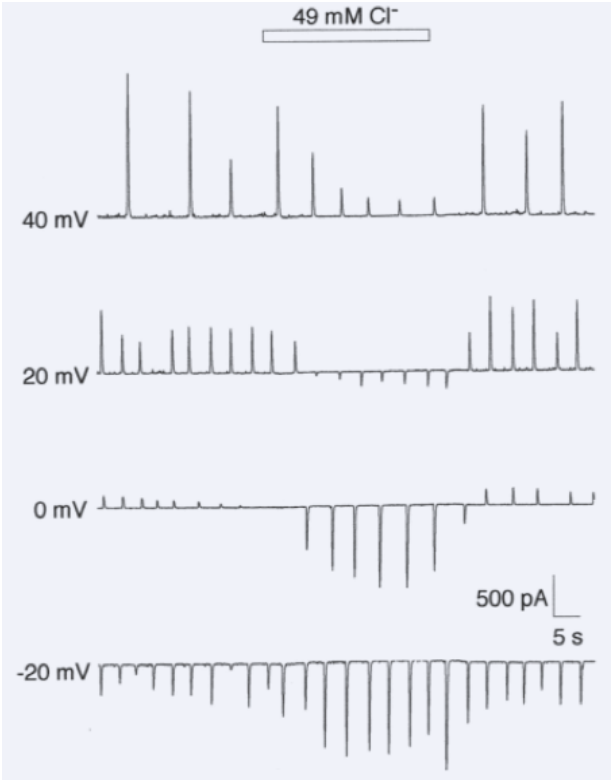

Fig. 3 Voltage clamp recording of STICs from an isolated ICC demonstrating that under control conditions (RHS of each trace) with 135 mM Cl^- in the path and pipette solution (predicted E_{Cl} = 0 mV) STICs reversed close to 0 mV. When E_{Cl} was shifted to +27 mV by reducing the Cl^- concentration in the bath solution to 49 mM by replacement with the non permeant anion, glutamate, the reversal potential shifted positive to +20 mV.

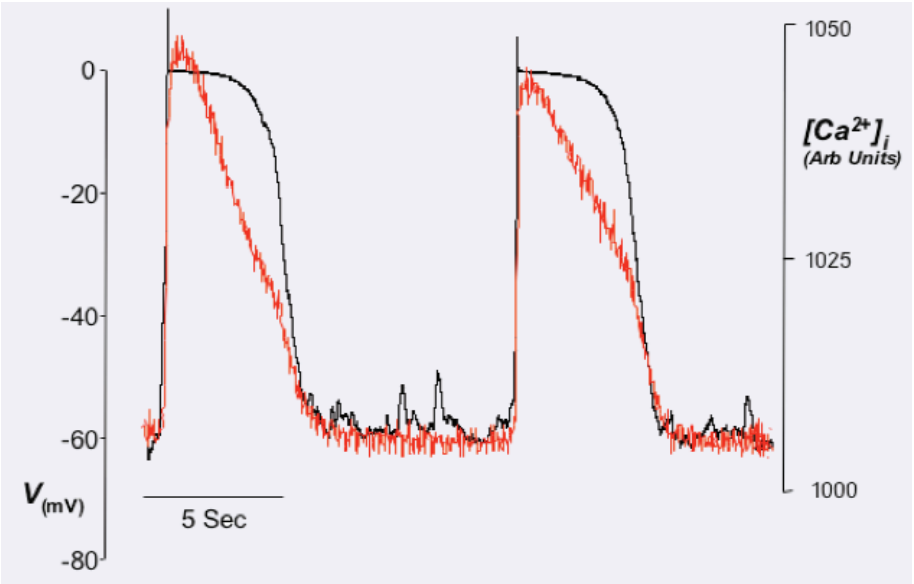

Fig. 4 Simultaneous recording of membrane potential in current clamp and $[Ca^{2+}]_i$ in a fluo-4 loaded ICC, imaged with a Nipkow spinning disk confocal microscope shows that STDs are associated with spontaneous Ca^{2+} oscillations.

driver of the pacemaker mechanism that initiated Ca^{2+}-induced Ca^{2+} release (CICR) at ryanodine receptors (RyR). This hypothesis was later tested by Johnston *et al*., in 2005 [32] and demonstrated that urethra ICC did indeed elicit regularly occurring Ca^{2+} waves, which were associated with STICs and STDs (see Fig. 4). These waves were abolished when RyR were blocked with ryanodine or tetracaine. In contrast, inhibition of IP_3 receptors (IP_3R) with 2APB did not abolish Ca^{2+} waves but severely disrupted their spatial spread. Therefore it appeared that the prime oscillator was actually the ryanodine sensitive store and not the IP_3 sensitive store as originally suggested by Sergeant *et al*., [30].

Role of Ca^{2+} influx

Johnston *et al*., [32] showed that the frequency of Ca^{2+} waves in urethra ICC was critically dependent on the external Ca^{2+} concentration, $[Ca^{2+}]_o$. For example, when $[Ca^{2+}]_o$ was reduced from 1.8 to 0.9 mM the frequency of Ca^{2+} waves fell by ~40%. Conversely when $[Ca^{2+}]_o$ was increased to 3.6 mM the frequency of the Ca^{2+} waves increased significantly. Finally, when Ca^{2+} was removed from the bath the oscillations stopped. A typical example of these effects is shown in Fig. 5. Although it was originally thought that these effects may have reflected changes in the Ca^{2+} content of the intracellular stores, Johnston *et al*., [32] demonstrated that 10 mM caffeine was able to evoke Ca^{2+} transients of similar amplitude in the presence of 1.8 and 0 mM $[Ca^{2+}]_o$. This suggested that the Ca^{2+} stores remained intact during this brief removal of $[Ca^{2+}]_o$ and pointed to the involvement of a direct contribution of Ca^{2+} influx to the pacemaker mechanism.

The involvement of several Ca^{2+} influx pathways to the pacemaker mechanism in urethral ICC has been evaluated. Since nifedipine (10 μM) failed to block either STICs at –60 mV or Ca^{2+} oscillations in unclamped cells [30, 32] it appears that L-type Ca^{2+} channels are not essential for pacemaking. Capacitative Ca^{2+} entry (CCE) also appears not be directly involved as blockers of it did not abolish STICs, suggesting that an alternative pathway must be involved [33]. A recent study by Bradley *et al.,* reported that Ca^{2+} influx *via* reverse Na^+/Ca^{2+} exchange (NCX) is of critical importance to the pacemaker mechanism in urethral ICC [34]. The idea that reverse NCX could be important stemmed from an observation by Putney *et al*., [35] who noted that cells which do not display CCE, utilize reverse NCX to replenish their Ca^{2+} stores. Bradley *et al*., [34] found that the selective reverse NCX inhibitors KB-R7943 and SEA 0400, significantly reduced the frequency of Ca^{2+} oscillations and STICs at –60 mV and decreased basal Ca^{2+}. However, it appears that these effects were not due to an effect on Ca^{2+} store replenishment as neither NCX inhibitor blocked Ca^{2+} release evoked by caffeine or noradrenaline. Instead, it appears that Ca^{2+} influx *via* this pathway acts to raise basal Ca^{2+} levels in urethra ICC sufficiently to activate RyR. Such a mechanism is rem-

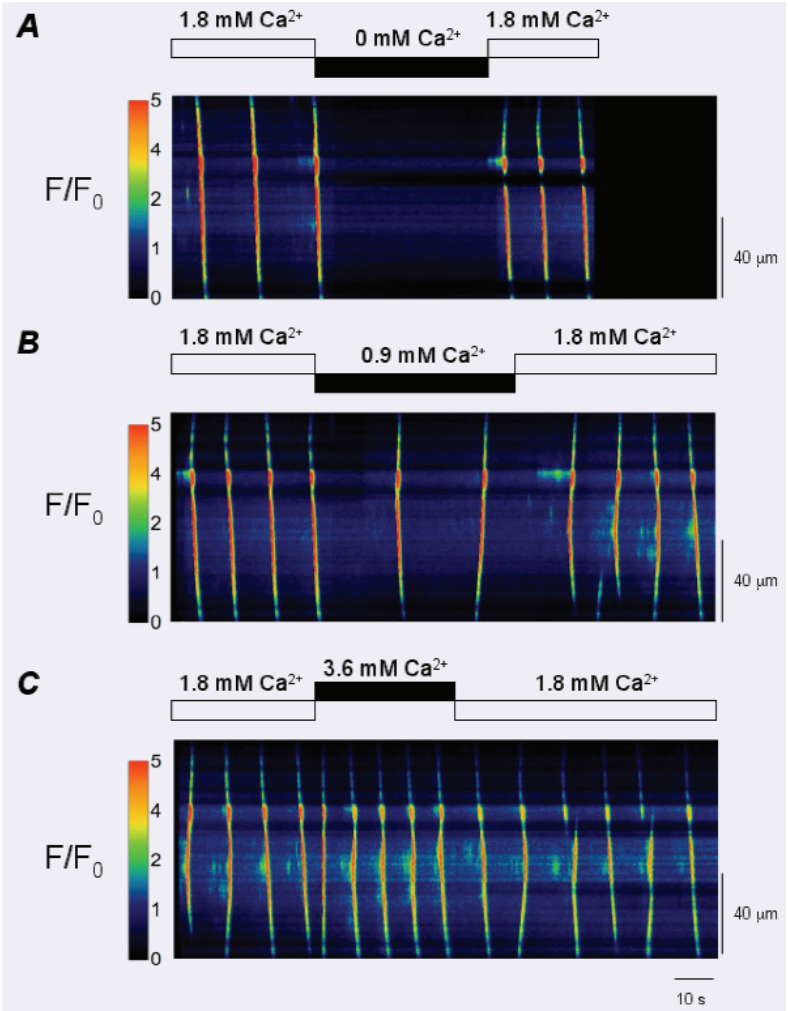

Fig. 5 Pseudo linescan or x, t plot of propagating Ca^{2+} waves in an ICC. Ca^{2+} waves were abolished by removal of $[Ca^{2+}]_o$ (**A**). Reduction in $[Ca^{2+}]_o$ from 1.8 to 0.9 mM reduced the frequency of Ca^{2+} waves by ~50% (**B**). An increase in $[Ca^{2+}]_o$ to 3.6 mM increased the frequency of Ca^{2+} waves (**C**).

iniscent of that reported by Leblanc & Hume [36] who found that ryanodine sensitive sarcoplasmic reticulum (SR) Ca^{2+} release in cardiac myocytes was triggered by Ca^{2+} entry *via* reverse NCX.

Modulation of pacemaker activity in urethral ICC

There is now convincing evidence which shows that ICC in the GI tract act as mediators of both inhibitory and excitatory neurotransmission. Studies using mutant mice lacking ICC (W/W^v) have impaired post-junctional neural responses compared to wild type controls [14–16]. These effects have been well documented elsewhere [2, 4–5], however it is interesting to consider the possibility that ICC in the urethra could also have a role in neurotransmission. Myogenic tone in the urethra is modulated by a number of excitatory and inhibitory neurotransmitters including noradrenaline, acetylcholine and nitric oxide, however the precise cellular mechanisms underlying these effects are still unclear. Several studies have shown that the frequency of slow waves in strips of urethra smooth muscle is modulated by neurotransmitters. Since ICC are thought to be the source of this activity, it is possible that the effects of neurotransmitters are mediated *via* these cells, as is the case in the GI tract.

Modulation of ICC by noradrenaline

Noradrenaline is the principal inhibitory neurotransmitter in the urethra [37] and it has been shown that myogenic tone is increased by application of α-adrenoceptor agonists [9]. It now appears that these effects are mediated by an upregulation of the

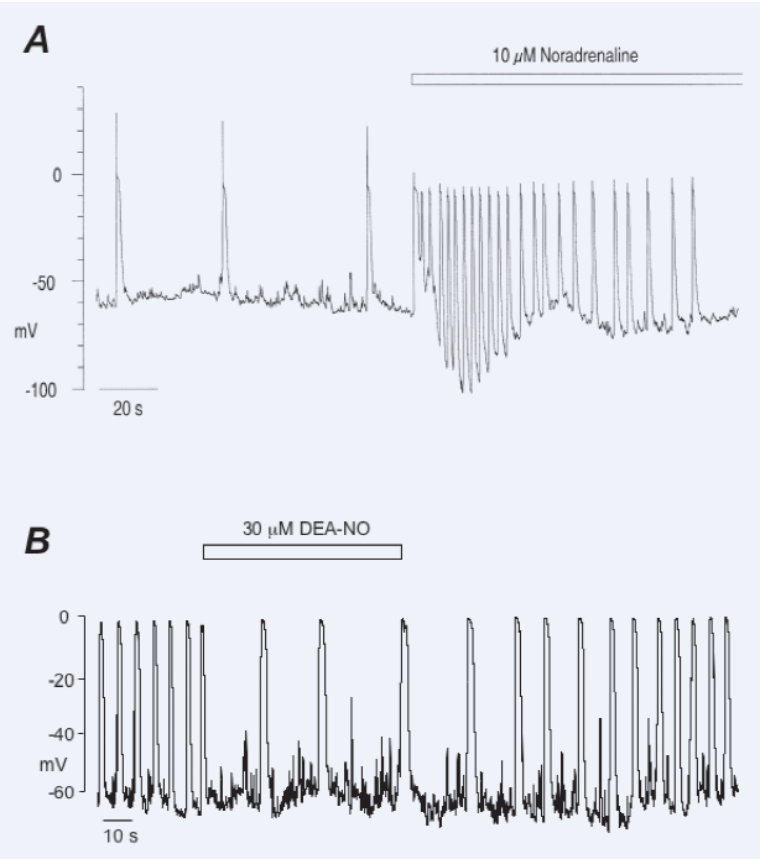

Fig. 6 Current clamp records from isolated interstitial cells demonstrating that the frequency of STDs in increased by noradrenaline (**A**) and decreased by the NO donor, DEA NO (**B**). *Reproduced from Sergeant et al., 2000 & Sergeant et al., 2006 respectively.*

underlying pacemaker mechanism as Hashitani *et al.,* [11] showed that exogenous application of noradrenaline increased the frequency of slow waves in the rabbit urethra. Sergeant *et al*., [38] investigated if isolated urethra ICC responded to exogenous application of noradrenaline in a similar fashion. Fig. 6A shows that application of noradrenaline to an isolated urethral ICC held under current clamp increased the frequency of STDs - an effect that was remarkably similar to that observed in the whole tissue [11]. These effects resulted from stimulation of α_1-adrenoceptors as they were abolished by phentolamine and prazosin. In addition, the noradrenaline responses were attenuated by CPA and 2APB as well as niflumic acid and A-9-C, suggesting that activation of α_1-receptors led to release of Ca^{2+} from IP_3 sensitive stores [39–41] and activation of ICl_{Ca}, as has been reported in other smooth muscle types [42].

To establish if ICC contributed to noradrenergic stimulation in intact strips of urethral smooth muscle, Sergeant *et al.,* [38] took advantage of the differential expression of ICl_{Ca} in smooth muscle cells and ICC in the rabbit urethra. This study used Cl^- channel blockers to selectively lesion the ICC and thus assess their contribution to α-adrenoceptor mediated neurogenic contractions. As Fig. 7 suggests, pharmacological ablation of urethral ICC with A-9-C led to a reduction in spontaneous and α-adrenoceptor mediated neurogenic contractions, consistent with the idea that the ICC contribute to excitatory neurotransmission.

Modulation of ICC by nitric oxide

As Fig. 8 suggests, an inhibitory response to nerve stimulation is unmasked in the urethra when the contribution of neurally released acetylcholine and noradrenaline is abolished pharmacologically. Fig. 8 shows a typical example of the effects of nerve stimulation at a variety of stimulus frequencies in a strip of rabbit urethra smooth muscle before and during incubation with atropine (1 μM) and guanethidine (1 μM). In contrast to previous reports [43–44], we find that the rabbit urethra, like the

human [45], pig [9] and sheep urethra [46] readily generates spontaneous tone in the absence of any drugs. During inhibitory nerve stimulation (lower panel Fig. 8) relaxations of myogenic tone were apparent in response to nerve stimulation at 0.5, 1, 2 & 4Hz. The neurotransmitter responsible for this relaxation in rabbit, human, rat and sheep urethra is nitric oxide, since incubation of the tissues with NO synthase blockers abolish the inhibitory response [47]. However, a number of studies have demonstrated that other NANC inhibitory neurotransmitters may also contribute to the neurogenic relaxations in the guinea-pig and pig [9, 48].

Smet *et al.,* [12] and Waldeck *et al.,* [43] presented the first studies to suggest that interstitial cells may be important in mediating neurally released NO responses. Their immunohistochemical studies demonstrated the presence of branched interstitial cells in human, guinea-pig and rabbit urethras respectively, that were immunopositive for cGMP following application of NO donors. A more recent study by Sergeant *et al*., [49] demonstrated that the electrical activity in urethra ICC was inhibited by NO donors and other activators of the cGMP/PKG pathway. Fig. 6B shows a typical example of the inhibitory effects of the NO donor DEA-NO on spontaneous electrical activity recorded from an isolated ICC held under current clamp. These effects were mimicked by the membrane permeant analogue of cGMP, 8-Br-cGMP, the guanylyl cyclase activator, YC1 and the PKG activator, 8-Br-Sp-PET-cGMP [49] and are consistent with the idea that activation of the NO/cGMP/PKG pathway is responsible for mediating the effects of NO in the urethra [50]. In an attempt to further understand how nitrergic stimulation inhibited STICs and STDs in urethral ICC, Sergeant *et al*., [49] examined the effects of NO on the spontaneous Ca^{2+} waves that underlie these electrical events. Interestingly, as Fig. 9 suggests, nitrergic stimulation failed to alter the fre-

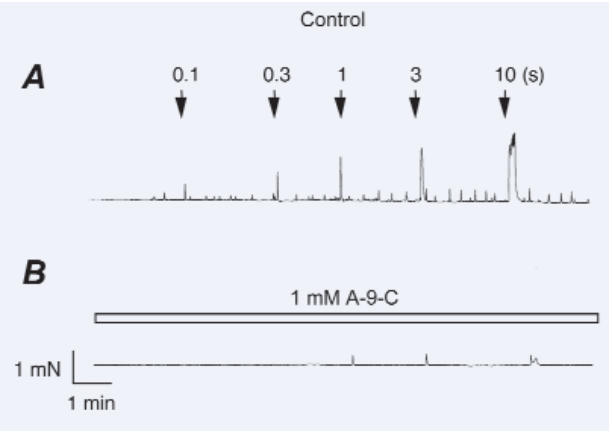

Fig. 7 EFS induced contractions of rabbit urethra smooth muscle at 10 Hz for durations of 0.1, 0.3, 1, 3 & 10 seconds are greatly reduced by 1 mM A-9-C. *Reproduced from Sergeant et al., 2001.*

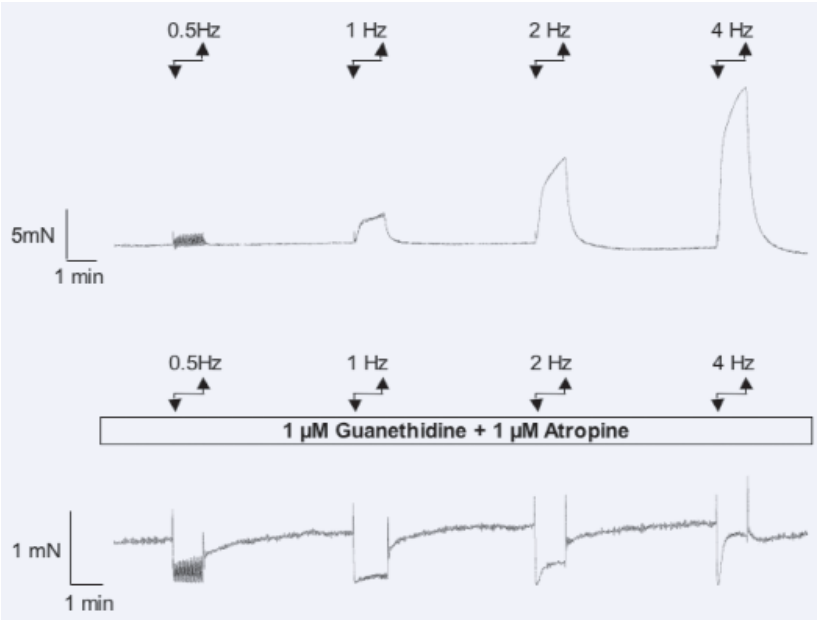

Fig. 8 Intramural nerve stimulation of strips of rabbit urethra leads to sustained elevations in tone. When the contribution of excitatory neurotransmitters is blocked by atropine and guanethidine (lower trace), neurogenic relaxations of basal tone are unmasked.

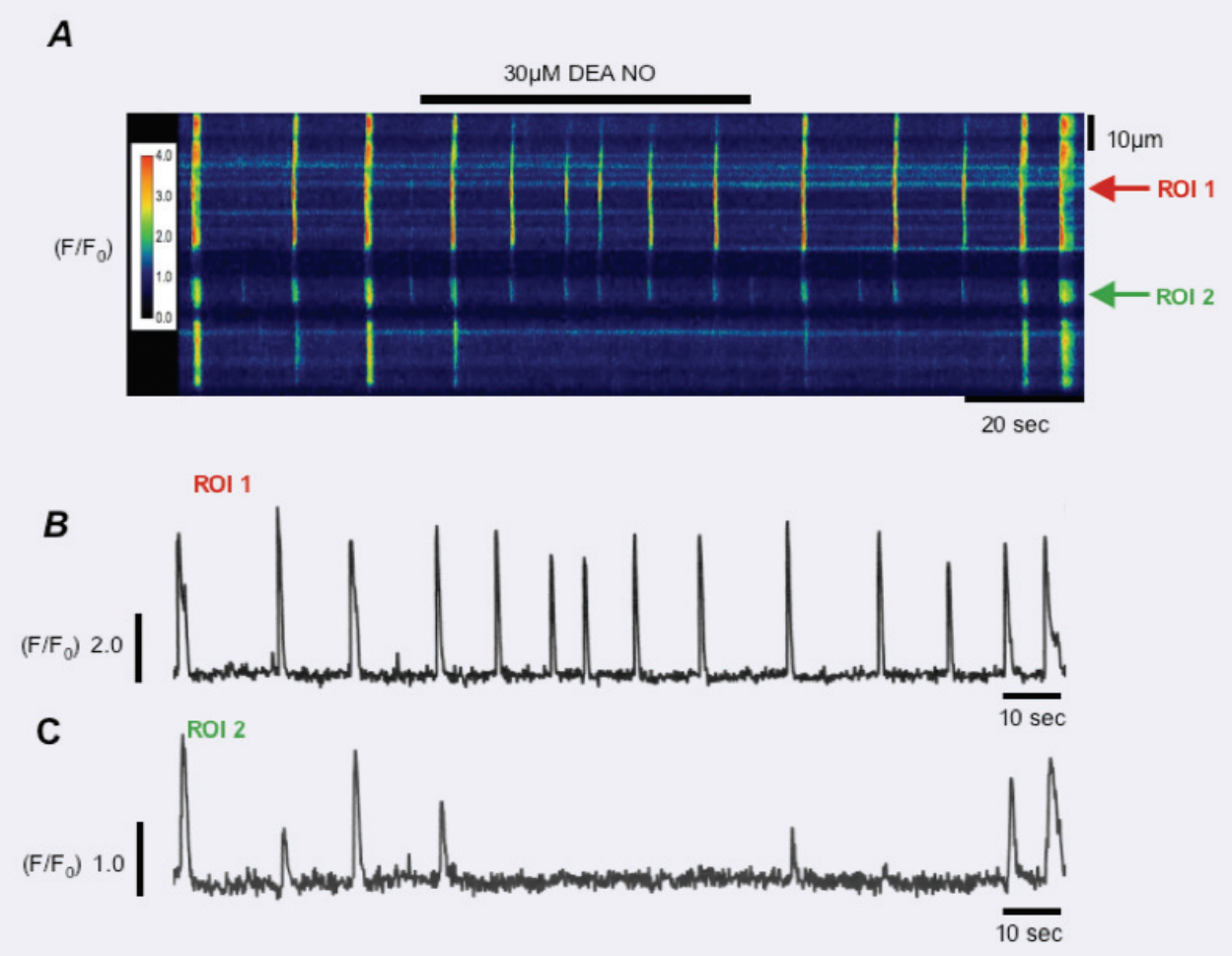

Fig. 9 DEA-NO reduces whole cell Ca^{2+} waves by reducing their spatial spread. Panel **A** shows a psuedo-linescan which demonstrates that DEA-NO reversibly reduced wave spread. In panel **B**, the oscillations at the site of origin were little affected by DEA-NO application, but they failed to spread to adjacent regions (**C**). *Reproduced from Sergeant et al., 2006.*

quency of the Ca^{2+} waves underlying the electrical activity. However, the distance over which the waves propagated was greatly reduced. Johnston *et al*., [32] had previously shown that inhibition of IP_3R with 2APB, uncoupled Ca^{2+} waves by decreasing their spatial spread, suggesting that IP_3R are involved with the propagation of Ca^{2+} waves. This prompted speculation that NO could affect release of Ca^{2+} from IP_3R. Consistent with this idea was the finding that noradrenaline evoked Cl^- currents were reduced by pretreatment with activators of the NO/cGMP/PKG pathway whereas caffeine evoked currents were unaffected. Therefore it appears that the inhibitory effects of NO on urethra ICC are due to decreased Ca^{2+} release *via* IP_3R. It is thought that the reduction in the spatial spread of the Ca^{2+} signal in urethral ICC by NO, would effectively limit the coupling between $[Ca^{2+}]_i$ and the Cl^- channels. They hypothesized that the reduced Ca^{2+} oscillations would fail to activate sufficient Cl^- channels to depolarize the ICC, resulting in inhibition of pacemaker activity and urethral tone.

Cholinergic excitation of ICC

Blockade of muscarinic receptors appears to have little effect on urethral tone *in vivo*, [51] or *in vitro* [46, 38] suggesting that neurotransmitter release

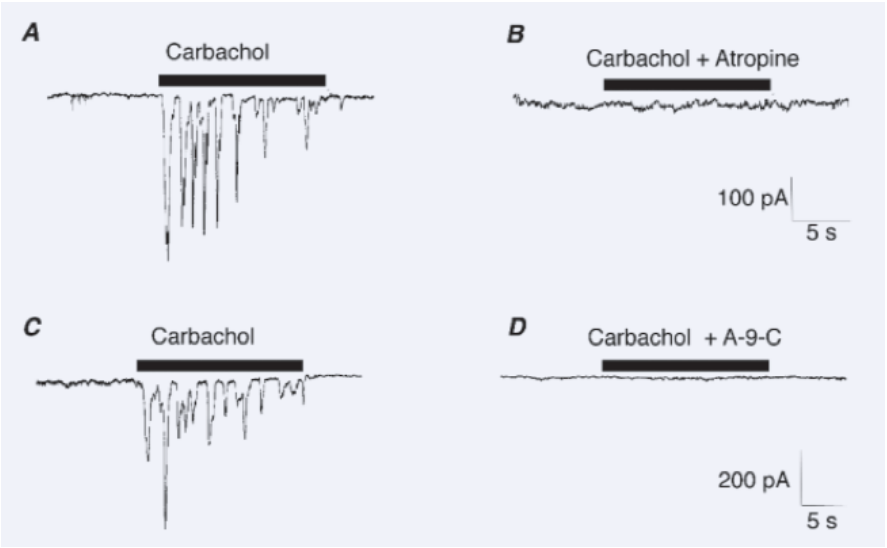

Fig. 10 Application of carbachol to isolated ICC voltage clamped at –60 mV induces transient inward currents (**A** & **C**) which were abolished by atropine (**B**) and A-9-C (**D**).

from cholinergic nerves contributes little to maintaining continence. However, stimulation of cholinergic nerves *in vitro* has been demonstrated to contract urethra smooth muscle in sheep, pigs, dogs and rabbits [45, 46, 52–54]. These effects appear to be mediated post-junctionally via activation of M_2 and/or M_3 muscarinic receptors [55, 56].

It is not yet known whether ICC contribute to cholinergic neurotransmission in the urethra. However, preliminary studies from our laboratory suggest that the frequency of STICs in freshly dispersed ICC is increased by application of muscarinic agonists. Fig. 10A shows a typical example of the effect of exogenous carbachol (10 μM) applied to an ICC held under voltage clamp at –60 mV. Under these conditions, carbachol evoked an initial large inward current followed by a series of smaller oscillatory inward currents of diminishing amplitude, which were reminiscent of the noradrenaline evoked currents demonstrated by Sergeant *et al.,* [38]. As Fig. 10B suggests, these excitatory effects were abolished in the presence of the muscarinic antagonist atropine (1 μM). Preliminary data shown from another cell in Fig. 10 C and D suggests that the carbachol induced currents were carried by Cl^- ions since they were abolished in the presence of A-9-C (1 mM). Future experiments will be directed towards establishing the receptor pharmacology and assessing if ablation of urethral ICC alters cholinergic neurotransmission in this tissue.

The studies noted above clearly show that ICC in the urethra are modulated by excitatory and inhibitory neurotransmitters in a fashion consistent with that in the whole tissue. Whether ICC are the actual targets for these neurotransmitters has not yet been discerned and requires further experimentation.

Summary and future perspectives

The finding that ICC are present in the smooth muscle of the urethra has opened up a new and exciting avenue for research into the mechanisms responsible for the generation and modulation of urethral tone and urinary continence. The idea that tone in the urethra is dependent on the frequency of pacemaker activity in a similar fashion to phasic muscles of the GI tract is novel and clearly warrants further investigation. We are at an early stage in our understanding of the true physiological role of these cells and the hypothesis that ICC are specialised pacemaker cells which influence surrounding smooth muscles, needs to be tested directly. Nevertheless, the data accumulated on single ICC has, in the last few years provided an excellent insight into the cellular basis of pacemaker activity in freshly dispersed cells. Although this approach has removed any complications associated with cell culture, the development of a specific urethral ICC culture system is essential. This would assist the development of molecular strategies to selectively lesion specific proteins involved in pacemaking. Future studies would also benefit from a more inte-

grative approach that permits the investigation of ICC under more physiological conditions.

Although the clinical significance of urethra ICC has yet to be determined, it has been reported that increased numbers of ICC-like cells are found in bladder tissues in patients who suffer from 'over-active' bladder [57]. Therefore, it will be interesting to investigate if ICC in the urethra are also associated with disorders of the urinary tract. If so, ICC may represent novel targets for the development of drugs to treat these stressful conditions.

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