Spontaneous Activity in Urethral Smooth Muscle

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Gerard P. Sergeant, Mark A. Hollywood, and Keith D. Thornbury

Abstract

The urethra is a muscular tube that extends from the bladder neck and is composed of an inner layer of smooth muscle referred to as the internal urethral sphincter and an outer layer of striated muscle which forms the external urethral sphincter. The smooth muscle layer can be separated into an inner layer of longitudinally orientated smooth muscle and an outer, relatively thinner, layer of circular muscle. Tonic contraction of both the smooth and striated muscle components of the urethra generates a urethral closure pressure which exceeds intravesical pressure in the bladder to maintain urinary continence. It is likely that contraction of urethral smooth muscle is involved in the long-term maintenance of tone, since it can achieve this at relatively low energy cost, whereas the striated muscle contributes more to the rise in urethral tone that accompanies increases in bladder pressure secondary to coughing or other sudden increases in intra-abdominal pressure. The level of urethral smooth muscle tone is regulated by several autonomic neurotransmitters, including noradrenaline, acetylcholine, ATP and nitric oxide. However, it is also clear that urethral smooth muscle is capable of generating significant tone in the absence of neural input. In this chapter we will discuss the mechanisms responsible for contraction of urethral smooth muscle, with specific focus on the role of ion channels and Ca^{2+} handling proteins to this process. The mechanisms underlying spontaneous activity in urethral interstitial cells (UICs), putative pacemaker cells of the urethra, will also be examined along with the modulation of these mechanisms by key excitatory and inhibitory neurotransmitters.

Keywords

Urethra · Smooth muscle · Pacemaker · Interstitial cells of Cajal · Calcium waves · STICs · STDs

6.1 Introduction

Urinary continence is maintained by the concerted actions of the bladder, urethra, pelvic floor muscles, and surrounding connective tissues. The bladder and urethra work as a functional reciprocal unit under normal conditions, such that during the storage phase, the detrusor muscle of the bladder remains relaxed while the urethra is

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contracted to allow gradual filling of the bladder with urine and prevent leakage. In contrast, during voiding, the urethra relaxes and the detrusor contracts to facilitate emptying of the bladder [1, 2]. Therefore, the primary functions of the urethra are to (1) maintain urinary continence by generating a closure pressure that exceeds intravesical pressure and (2) facilitate voiding of urine at micturition. The urethra is composed of an inner layer of smooth muscle referred to as the internal urethral sphincter and an outer layer of striated muscle which forms the external urethral sphincter. Both muscle layers function together as a sphincter complex, albeit with distinct roles. The striated muscle is thought to be important in resisting rapid increases in abdominal pressure that occur during coughing or laughing. In contrast, contraction of the smooth muscle layer is important for generating and maintaining sufficient urethral closure pressure during bladder filling to prevent leakage [1, 3, 4]. Indeed, Jankowski et al. concluded that 'urethral smooth muscle, in comparison with striated muscle, is capable of imparting a greater influence on the long-term functional responses of the urethra' [5].

Stress urinary incontinence (SUI) arises when intravesical pressure exceeds urethral pressure, in response to sudden increases of intra-abdominal pressure and is common in women after vaginal delivery child birth [6]. Traditionally, this was primarily viewed as being due to loss of urethral support, referred to as urethral hypermobility. However, it has been reported that SUI in women was more likely to be associated with a reduction in urethral resistance, rather than anatomical changes in urethral support [7, 8]. There is also evidence that this may arise through an effect on urethral smooth muscle, as Prantil et al. showed that in a rat model of acute SUI, induced by simulated birth trauma, there was reduced urethral smooth muscle tone, indicating that 'the basal smooth muscle tone acts as a prime coordinator to the continual maintenance of continence' [9]. Urethral closure pressure also declines with age [10, 11], and this is correlated with a decrease in the density of circular smooth muscle in the urethra [12]. Therefore, it is clear that urethral smooth muscle tone makes an important contribution to urinary continence and its dysfunction is associated with urinary incontinence.

The smooth muscle of the urethra can be separated into two layers consisting of an internal longitudinally orientated layer and an outer, relatively thin, circular muscle layer. While it is clear that contraction of circularly orientated smooth muscle could maintain continence by occluding the lumen of the urethra, the precise role of the longitudinal layer is still unclear [1]. Urethral smooth muscle (USM) generates spontaneous tone [13], which can be modulated by several autonomic neurotransmitters including nitric oxide [14, 15], noradrenaline [16, 17], adenosine triphosphate [18–21] and acetylcholine [14, 22–24]. Although there are many unanswered questions regarding the mechanisms that underlie urethral tone, a clearer picture of the cellular mechanisms that contribute to spontaneous activity in USM is beginning to emerge. It is now recognised that several cell types within the smooth muscle layer of the urethra are spontaneously active, including smooth muscle cells and a population of cells, namely urethral interstitial cells (UICs). UICs are also referred to variously as interstitial cells [25], interstitial cells of Cajal (ICC, by analogy with similar cells that have been well described in the gastrointestinal tract [26]), ICC-like cells [27], and Cajal-like interstitial cells [28]. In this chapter we will describe the mechanisms proposed to underlie spontaneous activity in USM and examine how it is modulated by autonomic neurotransmitters.

6.2 Spontaneous Activity in Urethral Smooth Muscle

In comparison with the detrusor and gastrointestinal tract, studies on smooth muscle from the urethra are relatively sparse. The lack of research on this tissue has been attributed to the small size of the urethra in rats, guinea-pigs and mice [29], but it may also reflect an historical underestimation of the functional importance of urethral smooth muscle in maintaining continence. Nevertheless, early intracellular microelectrode recordings by Callahan and Creed on strips of guinea-pig USM, found that it generated spontaneous electrical

activity which consisted of 'bursts of spikes' (once every 1–7 min) separated by quiescent periods [30]. There were 20–30 spikes observed within these bursts, which lasted for 7–10 s. The mean resting membrane potential in the quiescent periods was −42 mV. An interesting observation from this study was that the electrical activity in the USM was poorly coordinated and that there was 'limited spread of activity'. For example, 'if an electrode was withdrawn from one cell and inserted into another, less than 1 mm away, it was frequently found that the patterns of activity were out of phase'. They also reported that, in contrast to observations in the detrusor, it was not possible to record spikes evoked by extracellular current pulses in USM. They suggested that the limited spread of activity 'probably prevents generalised, synchronous contraction'. Later studies by Callahan and Creed [31] on rabbit USM found that spontaneous electrical activity consisted of regular, single or (occasionally) compound spikes occurring at a frequency of 9–30 per min [31]. They also subsequently reported that rabbit USM developed action potentials and spontaneous depolarisations of ~16 mV in amplitude [24].

A seminal study by Hashitani et al. further elucidated the mechanisms underlying spontaneous electrical activity in the urethra [32]. They made intracellular microelectrode recordings from the circular smooth muscle layer of rabbit urethra and found that it produced large, regularly occurring depolarisations, termed slow waves (SWs) as well as smaller, irregularly occurring events, termed spontaneous transient depolarisations (STDs) (Fig. 6.1). Both SWs and STDs were resistant to atropine, phentolamine, guanethidine and tetrodotoxin, indicating that they were not of neural origin. The L-type Ca^{2+} channel antagonist, nifedipine failed to block these events, however, they were inhibited by reducing external [Cl^-] and by application of the Ca^{2+}-activated chloride channel (CACC) blockers niflumic acid and DIDS, suggesting an involvement of CACCs in their generation. Hashitani et al. also found that SWs and STDs were inhibited by the SERCA (sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase) inhibitor, cyclopiazonic acid (CPA) as well as caffeine, indicating that Ca^{2+} released from intracellular stores was required for the generation of spontaneous electrical activity. These data indicated that the spontaneous electrical activity in USM was mediated by release of Ca^{2+} from intracellular stores which activated CACCs. Similar findings were also reported for guinea-pig USM, whereby STDs and slow waves were also abolished by niflumic acid, low chloride solution, CPA and caffeine [33].

The finding that tonic contraction of USM was associated with phasic electrical activity was not intuitive and was somewhat at odds with the prevailing view that spontaneous myogenic tone depended on continuous entry of calcium through L-type Ca^{2+} channels [13]. However, given the poor coupling of electrical activity observed in the urethra [30], it is possible that asynchronous electrical events and their associated contractions, sum to form an overall tonic contraction. This idea was explored in more detail in imaging studies of intact USM strips [27, 34].

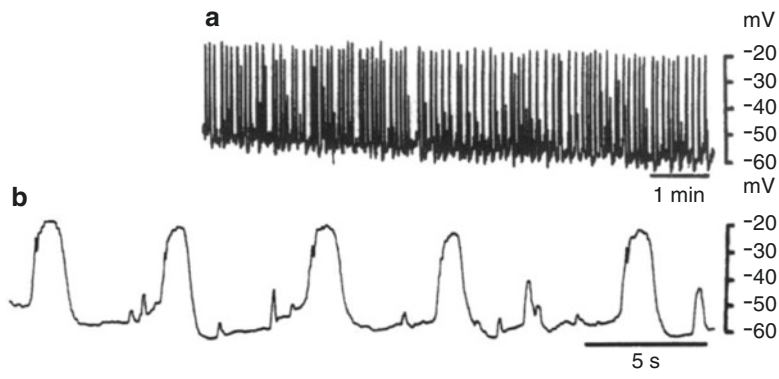

Fig. 6.1 Slow waves (SWs) and spontaneous transient depolarisations (STDs) recorded from the rabbit urethra. (**a**, **b**) Records from the same cell on different time scales. Adapted from [32]

Hashitani and Suzuki examined the properties of spontaneous Ca^{2+} transients recorded from both smooth muscle and interstitial cells of the rabbit urethra in situ [27]. The topic of interstitial cells in USM will be addressed later in this chapter but, for now, we will focus on spontaneous activity that has been recorded in urethral smooth muscle cells (USMCs). They reported that SMC in the rabbit urethra, loaded with fluorescent Ca^{2+} indicator, fluo-4 AM, generated spontaneous Ca^{2+} transients at a frequency of ~11 per min. These events occurred either as 'non-propagated Ca^{2+} transients or intercellular Ca^{2+} waves within a muscle bundle'. However, in contrast to intercellular Ca^{2+} waves observed in detrusor smooth muscle bundles of the guinea-pig bladder [35], the Ca^{2+} waves originating from a single site in USM often failed to spread across muscle bundles. When changes in muscle tension were measured simultaneously with intracellular [Ca^{2+}] it was found that 'there was no correlation between muscle contractions and Ca^{2+} transients in any particular muscle bundle within the preparations, presumably arising from a low synchronicity between bundles' [27].

Drumm et al. investigated this matter further by examining Ca^{2+} transients in USM strips dissected from SmMHC-Cre-GCaMP3 mice, which have a genetically encoded Ca^{2+} sensor, GCaMP3, selectively expressed in SMCs [34]. This study revealed that USMCs within smooth muscle bundles fired spontaneous intracellular Ca^{2+} transients. However, while distinct Ca^{2+} events could be imaged within individual USMC there was no evidence of intercellular propagation of Ca^{2+} events within USM bundles. Indeed, spontaneous Ca^{2+} events that occurred in adjacent SMCs were not synchronised and appeared to be independent of the activity occurring in their neighbouring cell. Furthermore, it was also apparent that the firing of intracellular Ca^{2+} waves in USMCs was associated with small contractions of individual USMC in muscle bundles. These contractions, like the intracellular Ca^{2+} waves, occurred asynchronously and failed to spread cell-to-cell across the muscle bundles.

Therefore, electrical and imaging studies of intact USM strips indicate that USM develops spontaneous depolarisations and Ca^{2+} transients, respectively, but that these events are poorly coupled, resulting in asynchronous contractions across the tissue that sum to give an overall tonic contraction. The cellular mechanisms underlying the spontaneous activity described above are discussed below.

6.3 Spontaneous Activity in Isolated Urethral Smooth Muscle Cells

Ca^{2+} waves recorded from rabbit USM, in situ, appear to rely on a combination of Ca^{2+} release from stores and Ca^{2+} influx via L-type Ca^{2+} channels, since they were inhibited by the SERCA inhibitor, CPA, the ryanodine receptor (RyR) antagonist, ryanodine, the inositol trisphophate receptor (IP_3R) inhibitor, 2-APB and the L-type Ca^{2+} channel antagonist nifedipine [27]. The involvement of L-type Ca^{2+} channels was consistent with other reports showing that nifedipine inhibited spontaneous action potentials and spikes superimposed on slow spontaneous depolarisations in rabbit USM [36, 37] and that application of 60 mM $[K^+]_o$ solution evoked robust Ca^{2+} transients and contractions in isolated rabbit USMCs [38]. Ca^{2+} waves in intact murine USM strips were also inhibited by Ca^{2+} store release modulators, including the SERCA inhibitor, thapsigargin, the RyR antagonist, tetracaine and the IP_3R inhibitor, xestospongin C [34]. Surprisingly however, Ca^{2+} waves in murine USMCs were resistant to the L-type Ca^{2+} channel modulators nifedipine, nicardipine, isradipine and FPL, but were abolished by Ca^{2+}-free bathing media, the store operated Ca^{2+} entry channel (SOCE) blocker SKF 96365 and the Orai channel antagonist, GSK-7975A [34]. Therefore, it appears that, in contrast to rabbit USMCs, Ca^{2+} waves in murine USMCs are not reliant on Ca^{2+} influx via L-type Ca^{2+} channels.

To date, only a few labs have made patch clamp recordings from freshly isolated USMCs. One of the main contributors to this field was Teramoto and colleagues in Alison Brading's Lab in Oxford. They systematically characterised

K^+_{ATP} channels in USM and showed that these channels have an important functional role in regulating membrane potential and relaxation of this tissue [39, 40]. The nature and molecular identity of K^+_{ATP} channels in USM has been comprehensively reviewed elsewhere [29, 41] and therefore will not be recapitulated here.

The intracellular microelectrode recordings by Hashitani et al. indicated that STDs and slow waves recorded from intact rabbit USM strips arose from activation of CACCs [32]. The first study to examine CACC currents (I_{ClCa}) in isolated USMCs was by Cotton et al. [42]. Using the perforated patch configuration of the whole cell voltage clamp technique, they found that freshly isolated SMC from the sheep urethra exhibited robust I_{ClCa}. Furthermore, they found that a proportion of these cells were spontaneously active, developing STDs and action potentials under current clamp that were sensitive to the CACC inhibitor anthracene-9-carboxylic acid (A-9-C, Fig. 6.2). These findings led the authors to propose that CACC in sheep USMCs may function as a pacemaker current. Later investigation of these cells found that ~10% of sheep USMCs developed spontaneous transient inward currents (STICs) when maintained at −60 mV [43]. These events were inhibited by the CACC blockers niflumic acid and A-9-C, and reversed near E_{Cl}, confirming the importance of CACCs to spontaneous activity in USM. STICs in sheep USMCs were also inhibited by ryanodine and caffeine suggesting that Ca^{2+} release from intracellular stores was important for activation of these events. Sancho et al. found that Anoctamin 1 (Ano1), also referred to as TMEM16A, and now recognised as the molecular correlate of CACCs [44–46], was expressed in the smooth muscle layer of the sheep urethra, consistent with idea that Ano1 encoded CACCs in sheep USMCs [47]. This study also reported Ano1 expression in USM of rats and mice, although Huang et al. (2009) failed to detect Ano1 in murine USM [48]. Precise reasons for the divergent findings in these studies is unclear, although differences in antibody specificity and experimental protocols have been suggested [47].

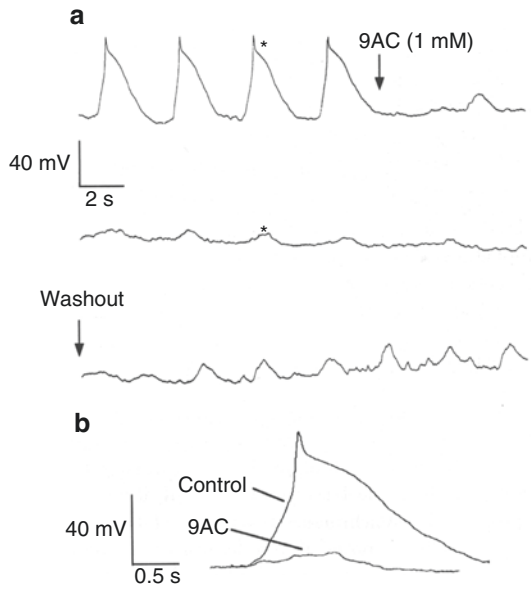

Fig. 6.2 Effect of A-9-C on spontaneous electrical activity in sheep urethral SMC. (**a**) Top panel, shows recordings made in current clamp using a Cs^+-filled pipette before addition of A-9-C (1 mM). The top two traces are a continuous recording. (**b**) events marked by * in **a** are compared on an expanded time scale, showing blockade of the action potential and reduced amplitude of the STD. Adapted from [42]

In order to further explore the cellular basis of the spontaneous activity described by Hashitani et al., in the rabbit urethra, Sergeant et al. undertook a patch clamp investigation to determine the properties of SMC freshly isolated from the rabbit urethra [25, 32]. Surprisingly however, despite having robust L-type Ca^{2+} currents, evidence for I_{ClCa} in these cells was weak. The large slowly developing inward currents and tail currents, typical of I_{ClCa}, that were evident in sheep USMCs, were not readily resolvable in rabbit USMCs. Current-voltage (IV) data averaged from 21 SMCs demonstrated that these cells had only small, sustained, inward currents at −30 mV and which reversed close to E_{Cl}. Furthermore, only ~3% of the rabbit SMC developed spontaneous electrical activity, even though they could respond to depolarising current injection (40 ms) by producing action potentials [25]. A later study by Hollywood et al. examined inward currents in SMCs isolated from the human proximal urethra using the whole cell patch clamp technique, and while L and T-type Ca^{2+} currents were reported,

there was no evidence of I_{ClCa} [49]. However, this subject warrants further investigation, using the perforated patch configuration of the whole cell patch clamp technique to optimise the conditions required to detect I_{ClCa}. Therefore, at the present time, there is still a lack of consensus regarding the contribution of CACCs in USMCs to spontaneous activity in intact USM strips. However, an interesting observation by Sergeant et al. was that although the evidence for I_{ClCa} in rabbit USMCs was weak, there was another cell type, termed 'interstitial cells' (referred to in this review as urethral interstitial cells, UICs) which had a robust I_{ClCa} and were spontaneously active [25].

6.4 Spontaneous Activity in Urethral Interstitial Cells

The first evidence that the urethra may contain a novel population of interstitial cells came from a study by Smet et al., which described a population of interstitial cells in human urethra preparations that were immuno-positive for vimentin and cGMP and bore a striking resemblance to the ICC described in the GI tract [50]. Smet et al. suggested that the UIC 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 [51–54]. However, Smet et al. did not propose a role for these cells in the generation of spontaneous electrical activity in USM. Interestingly however, Hashitani et al. did allude to the possibility that ICC could be involved in the development of SWs in USM by noting that interstitial cells of Cajal were mandatory for the development of SWs, in large tissues of the GI tract, but that such cells had not been discovered in USM [32].

Enzymatic dispersal of rabbit USM not only yielded SMCs, as noted above, but also a small population of branched, non-contractile cells that were darker and thinner than the SMCs [25], and were similar in appearance to freshly isolated ICC from the canine proximal colon [55]. These cells were immuno-positive for the intermediate filament vimentin and possessed several ultrastructural characteristics of GI ICC, including an incomplete basal lamina, abundant caveolae, abundant mitochondria, a well-developed smooth endoplasmic reticulum and a sparse rough endoplasmic reticulum. More than 80% of UICs were spontaneously active, generating STDs under current clamp and STICs when voltage clamped at −60 mV. These events reversed close to E_{Cl} and were blocked by the traditional CACC inhibitors, niflumic acid and A-9-C [25, 26]. More recently, Fedigan et al. [56] demonstrated that STICs and STDs in UICs were also sensitive to the TMEM16A channel inhibitors, $T16A_{inh}$-A01 and $CACC_{inh}$-A01, indicating that spontaneous electrical activity in UICs is likely to result from activation of TMEM16A channels (Fig. 6.3). Fedigan et al. also showed that the TMEM16A inhibitors reduced the level of spontaneous tone and the amplitude of nerve-evoked contractions of rabbit USM strips, indicative of an important functional role for these channels in urethral contraction [56].

Spontaneous activity in UIC is also dependent on Ca^{2+} release from intracellular stores as the STICs were abolished by the SERCA inhibitor CPA and were also sensitive to the RyR inhibitor, ryanodine, the phospholipase C (PLC) inhibitor, NCDC and the IP_3R inhibitor, 2-APB [57]. When UIC were held under voltage clamp at ~−30 mV and studied with K^+-rich pipettes, these cells also developed spontaneous transient outward current (STOCs). These STOCs could be categorised into two broad groups, based on their duration; (i) 'slow' STOCs (~1 s duration) that were coupled with STICs and (ii) 'fast' STOCs (<100 ms duration) that occurred independently of slow STOCs and STICs. Interestingly, while all events were abolished when RyRs were inhibited, only the slow STOCs and STICs were sensitive to 2-APB (Fig. 6.4). This observation led the authors to hypothesise that 2-APB-sensitive STICs and 'slow' STOCs arose from global Ca^{2+} events involving IP_3Rs, such as propagating Ca^{2+} waves, whereas the fast STOCs arose from RyR-dependent, localised Ca^{2+} events, such as Ca^{2+} sparks [57]. This idea was largely corroborated by Johnston et al. who, using a Nipkow spinning disc confocal microscope, reported that UIC

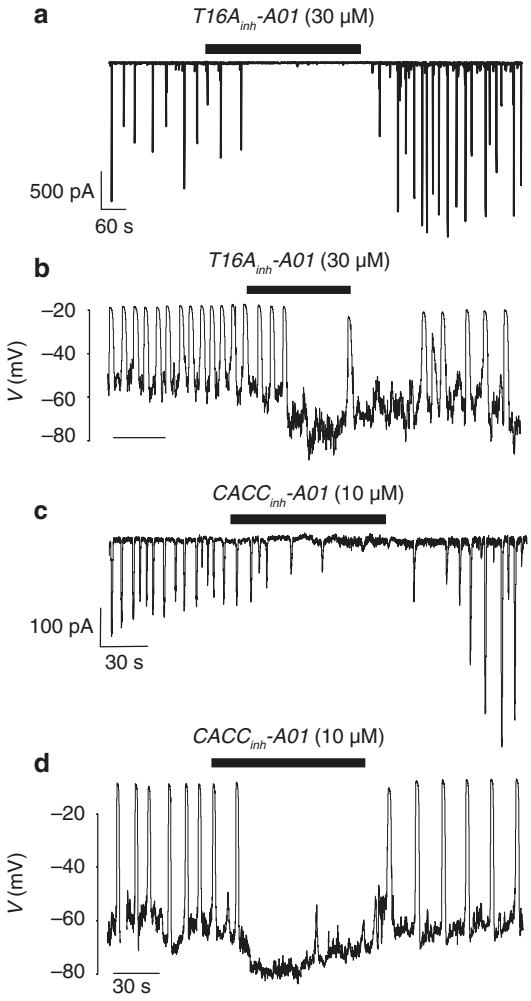

Fig. 6.3 Effect of CACC/TMEM16A inhibitors, *T16A$_{inh}$-A01* and *CACC$_{inh}$-A01*, on STICs (**a**, **c**, respectively) and STDs (**b**, **d**, respectively) recorded from freshly isolated rabbit UICs. Adapted from [56]

loaded with Fluo4-AM, exhibited spontaneous Ca^{2+} waves [58]. Simultaneous voltage clamp and Ca^{2+} imaging experiments revealed that STICs were associated with Ca^{2+} waves (Figs. 6.5 and 6.6). Application of 2-APB abolished STICs, but only reduced the spatial spread of Ca^{2+} waves [58]. In contrast, inhibition of RyRs with tetracaine, or ryanodine, abolished all spontaneous Ca^{2+} events. These findings prompted the idea that propagation of Ca^{2+} waves, and subsequent activation of STICs, required Ca^{2+} release from IP_3Rs, but that the initiation of these events was dependent on Ca^{2+} release via RyRs. In this model, Ca^{2+} release from RyRs was proposed as the 'prime oscillator'.

In addition to Ca^{2+} release from intracellular stores, it is clear that spontaneous activity in UIC is also dependent on Ca^{2+} influx across the plasma membrane [25, 27, 58]. STDs and spontaneous Ca^{2+} waves in UIC were abolished by removal of external Ca^{2+} and the frequency of Ca^{2+} waves was correlated with the external Ca^{2+} concentration ($[Ca^{2+}]_o$) as a reduction in $[Ca^{2+}]_o$ from 1.8 to 0.9 mM decreased Ca^{2+} wave frequency by ~40%, whereas an increase in $[Ca^{2+}]_o$ to 3.6 mM enhanced their frequency [58]. Similar findings were reported for UIC in situ [27]. Figure 6.6 is a representative trace showing the effect of Ca^{2+} removal on STICs and spontaneous Ca^{2+} waves, recorded simultaneously, in an isolated UIC. However, the mechanisms underlying Ca^{2+} influx in UIC and their interaction with Ca^{2+} release mechanisms has only recently been clarified.

Isolated UIC displayed robust L-type Ca^{2+} currents in response to step depolarisation [25];

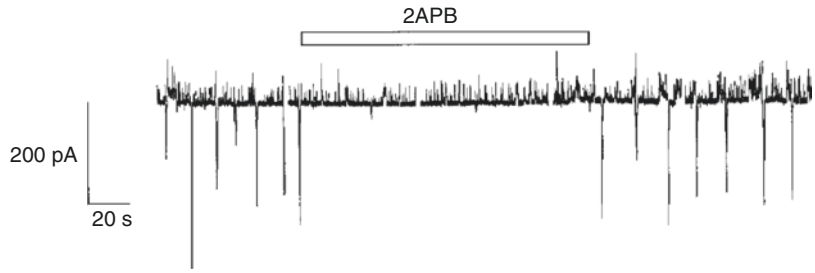

Fig. 6.4 The IP_3R inhibitor 2APB (100 μM) inhibits STICs and 'slow' STOCs, but not 'fast' STOCs in freshly isolated rabbit UIC voltage clamped at −30 mV. Adapted from [57]

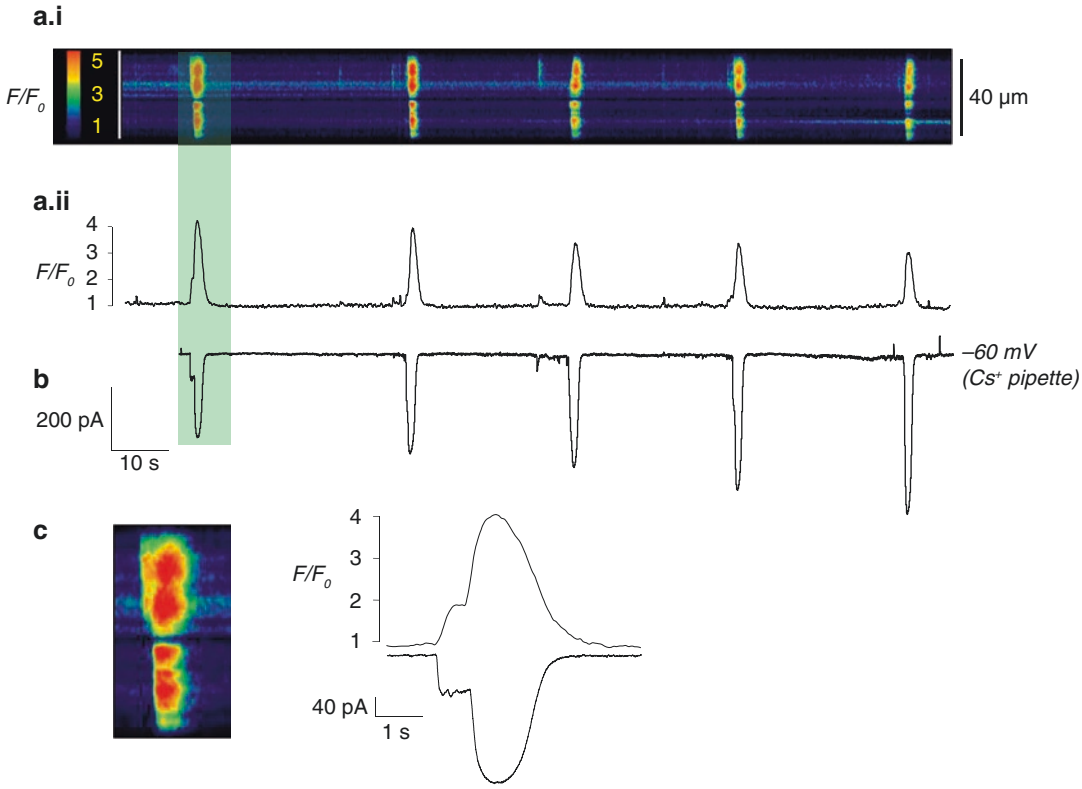

Fig. 6.5 STIC*s* in freshly isolated rabbit UICs are associated with spontaneous Ca^{2+} waves. (**a.i**, **a.ii**) Show a pseudo linescan image and corresponding intensity profile plot, of spontaneous Ca^{2+} waves in a rabbit UIC loaded with fluo4-AM. (**b**), is a simultaneous voltage clamp recording at −60 mV, showing that STICs are associated with the spontaneous Ca^{2+} waves. (**c**) Shows the Ca^{2+} wave and STIC depicted in the highlighted area in **a.i**, **a.ii** and **b** on an expanded time scale

however, STICs recorded at −60 mV in the same cell type were resistant to L-type Ca^{2+} channel blockade with nifedipine [57] or *D-cis* diltiazem [59], demonstrating that spontaneous activity in rabbit UICs is not reliant on Ca^{2+} influx via L-type Ca^{2+} channels. Similarly, spontaneous Ca^{2+} waves in non-voltage-clamped UICs were also unaffected by nifedipine [27, 58], consistent with this idea. Johnston et al. also noted that although spontaneous Ca^{2+} waves were abolished in Ca^{2+}-free media, caffeine-induced Ca^{2+} transients remained intact, suggesting that the abolition of spontaneous activity by removal of external Ca^{2+}, was not a function of Ca^{2+} store depletion. Consistent with this idea were the findings of Bradley et al. who found that although UICs exhibited a robust capacitative Ca^{2+} entry signal upon Ca^{2+} store depletion, blockers of this pathway, including Gd^{3+} (10 µM) and La^{3+} (10 µM), did not abolish spontaneous activity [60].

It now appears that one of the key Ca^{2+} influx pathways that drives spontaneous activity in UICs is via reverse mode-sodium calcium exchange (NCX). The selective reverse NCX inhibitors KB-R7943 and SEA0400 [61–64] significantly reduced both the frequency of Ca^{2+} oscillations and STICs in isolated rabbit UICs, and the level of spontaneous tone in intact USM strips [65]. Furthermore, a reduction in extracellular Na^{+} levels (to promote reverse mode NCX) increased the frequency of Ca^{2+} waves in UICs. Drumm et al. [66] investigated this issue further by examining the effect of the NCX inhibitors, as well as application of Ca^{2+}-free solution, on Ca^{2+} waves recorded using faster acquisition rates (50–97 FPS) than those employed in earlier stud-

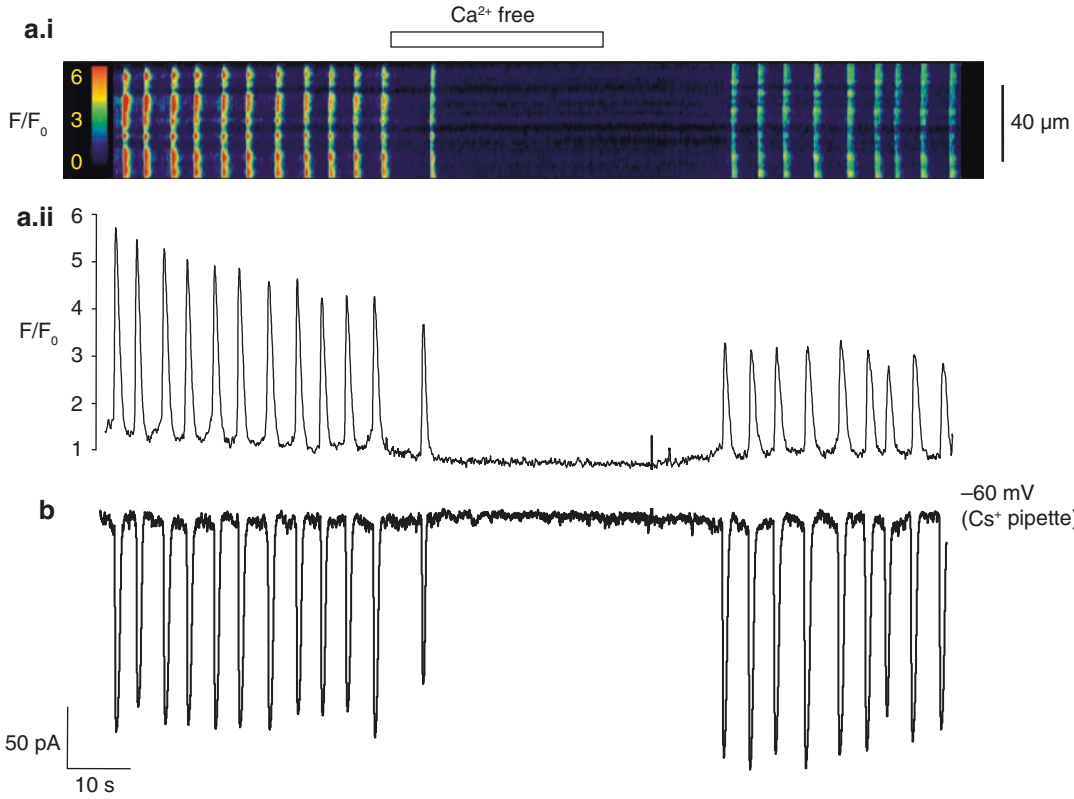

Fig. 6.6 Simultaneous recording showing that STICs and spontaneous Ca^{2+} waves in freshly isolated rabbit UICs are abolished by removal of extracellular Ca^{2+}. (**a.i**, **a.ii**) Show a pseudo linescan image and corresponding intensity profile plot, of spontaneous Ca^{2+} waves in a rabbit UIC loaded with fluo4-AM. (**b**) Simultaneous voltage clamp recording at −60 mV, showing that STICs are abolished by removal of extracellular Ca^{2+}

ies [58, 65]. In contrast to these studies, Drumm et al. [66] found that application of Ca^{2+}-free solution, KB-R7943 and SEA0400 (Fig. 6.7) abolished propagating Ca^{2+} waves, but unmasked brief, localised Ca^{2+} sparks that were not detected in the earlier studies [58, 65]. These effects were very similar to those induced by blockade of IP_3Rs with 2-APB (Fig. 6.7) suggesting that the role of Ca^{2+} influx via reverse NCX was to sensitise IP_3Rs to Ca^{2+}, which allows localised Ca^{2+} release events from RyRs to be converted into propagating Ca^{2+} waves.

Another Ca^{2+} influx pathway that has been proposed to regulate spontaneous activity in UICs is via cyclic nucleotide-gated (CNG) channels [59]. CNG channels are permeable to Na^+ and Ca^{2+} and their activation can induce local changes in cytosolic Ca^{2+} levels in response to a rise in cAMP or cGMP. $CNGA_1$ and $CNGB_1$ subunits, which form functional $CNGA_1$, or rod retinal-like CNG channels, were strongly expressed in a subpopulation of vimentin-positive ICC of the rat urethra, whereas only weak and diffuse CNG1A-immunoreactivity was evident in rat USMCs [67]. Furthermore, inhibition of CNG channels, with *L-cis* diltiazem, reduced the frequency of STICs and Ca^{2+} waves in rabbit UIC [59], however, the precise contribution of CNG channels to spontaneous activity in UIC is still unclear and requires further investigation.

Ward et al. suggested that mitochondrial Ca^{2+} handling may regulate the frequency of pacemaker activity in GI muscles [68]. This was based on the observations that inhibition of the electrochemical gradient across the inner mitochondrial membrane with the mitochondrial uncouplers

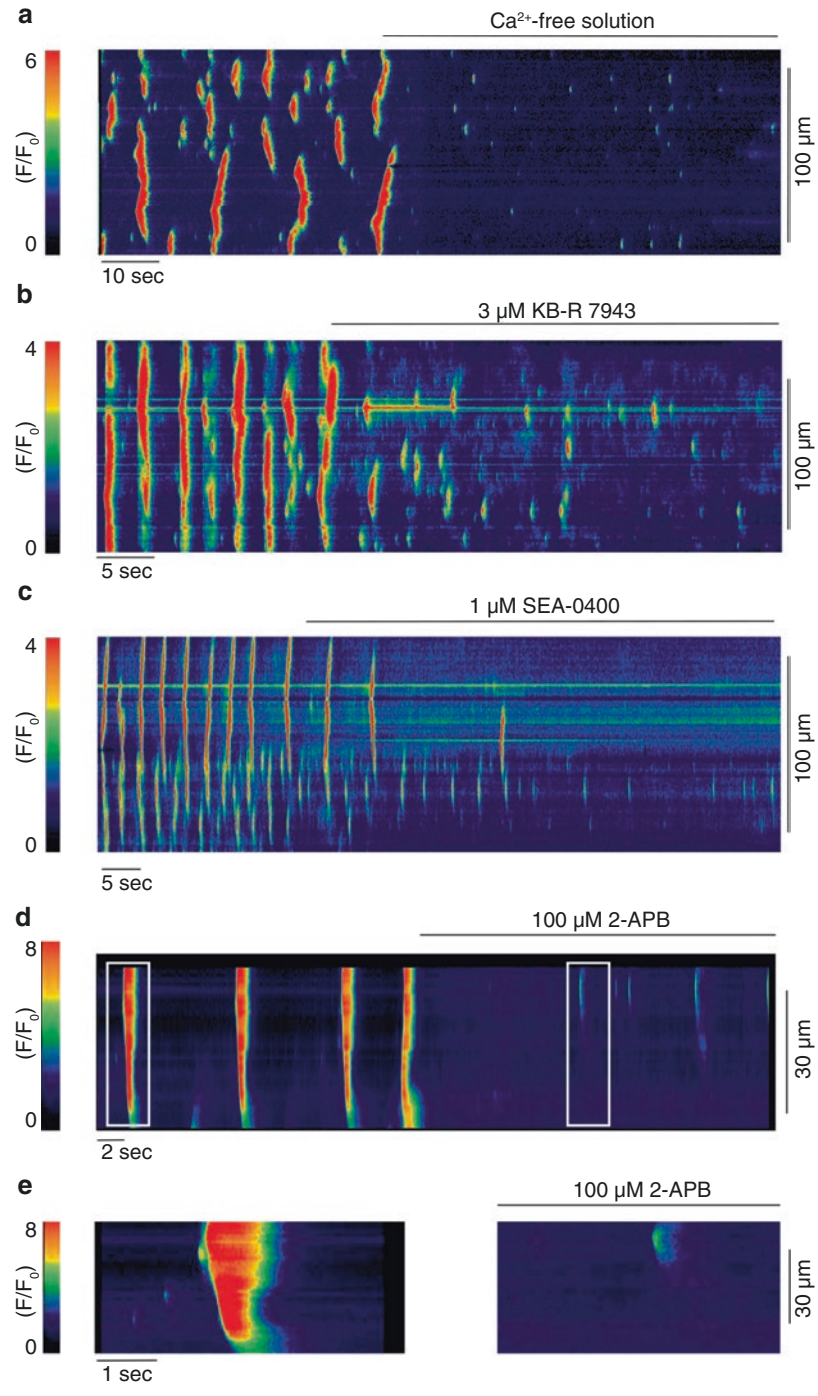

Fig. 6.7 Representative pseudo linescan images showing effects of application of Ca^{2+}-free external solution (**a**), the reverse NCX blockers KB-R 7943 (**b**) and SEA-0400 (**c**) and the IP_3R inhibitor 2-APB (**d**) on Ca^{2+} events, recorded at fast acquisition rates (50–97 FPS), in freshly isolated rabbit UICs. (**e**) Shows the Ca^{2+} events highlighted by the white boxes in **d** (before and during the presence of 2-APB) on an expanded time scale. Adapted from [67]

FCCP and CCCP or the respiratory chain (complex III) inhibitor antimycin, inhibited pacemaker currents in cultured ICC and blocked slow wave activity in intact GI muscles from mouse, dog and guinea-pig. Sergeant et al. reported similar findings on Ca^{2+} waves and STICs recorded from freshly isolated rabbit UICs [69] and Hashitani et al. also reported that CCCP could inhibit spontaneous Ca^{2+} waves in rabbit UICs [70]. These data indicated that spontaneous activity in UICs

may also be dependent on Ca^{2+} handling by mitochondria, as well as on Ca^{2+} release from the sarcoplasmic reticulum and Ca^{2+} influx across the plasma membrane. However, a recent study showed that antimycin blocked TMEM16A currents and that CCCP inhibited $Ca_V3.2$ currents [71]. Therefore, firm conclusions on the role of mitochondrial Ca^{2+} handling, based on use of these agents, should be treated with caution and further investigation is required to determine the contribution of mitochondrial Ca^{2+} handling to spontaneous activity in UIC.

Overall, it is clear that the smooth muscle layer of the rabbit urethra possesses a population of interstitial cells (UICs), which resemble ICC pacemaker cells in the GI tract, and which exhibit a pattern of spontaneous activity that is similar in nature to that recorded from intact strips of rabbit USM [25, 26, 72]. These observations prompted suggestions that UIC may function as pacemaker cells in the urethra. However, this hypothesis still requires further investigation. To date, there has only been one study that has simultaneously measured spontaneous activity in UICs and smooth muscle cells in USM. Hashitani and Suzuki were able to record Ca^{2+} transients in UICs and USMCs in tissue strips loaded with Fluo4-AM [27]. They found that Ca^{2+} transients recorded from rabbit UICs in situ were similar in nature to those recorded in isolated UICs [58]. Thus, they were abolished by application of CPA, ryanodine, caffeine and 2-APB and by removal of extracellular Ca^{2+}, but were resistant to application of nifedipine. On some occasions (21 preparations) spontaneous Ca^{2+} transients in USMCs were observed simultaneously with those of UICs within a field of view. Interestingly, in five of these preparations, UICs and USMCs generated synchronous Ca^{2+} transients with close temporal correlation between the signals in the two cell types, indicative of a degree of synchronicity between these cells (Fig. 6.8). In the remaining 16 preparations, USMCs generated Ca^{2+} transients independently from UICs, albeit at a lower frequency than those in the UICs. In contrast, when pairs of UICs were visualised in the same field of view, synchronous Ca^{2+} transients were observed in 17 out of 22 preparations, indicative a high degree of coupling between UICs. These authors concluded that '*UICs may act as a primary pacemaker in generating spontaneous contractions of USM. However, signal transmission from UICs to USMCs may be much less extensive than that between ICC and smooth muscle cells in the GI tract, and thus electrical pacemaking signals generated by UICs may be less securely transmitted' to smooth muscles*' [27]. Thornbury et al. reached similar conclusions, noting that 'there are multiple pacemakers within the urethra, but unlike the GI tract, these are not well networked' [73]. Therefore, it is apparent that both USMCs and UICs are capable of generating spontaneous activity but, in the rabbit urethra, at least, it appears that spontaneous activity originating from UICs is dominant and forms the basis of pacemaker in the intact tissue. Definitive determination of UIC function in the future will be dependent upon determining the identity of these cells by the presence of selective markers and examining tissue function when these cells are lesioned.

6.5 Modulation of Spontaneous Activity in UIC by Autonomic Neurotransmitters

6.5.1 Nitric Oxide

The principal inhibitory neurotransmitter in the urethra is Nitric Oxide (NO [14, 15]). NO is thought to exert its effects by activating the cGMP/protein kinase G (PKG) pathway, since electrical field stimulation (EFS) of inhibitory nerves or exogenous application of NO can elevate cGMP levels in this tissue [74–76], and neurogenic urethral relaxations are significantly attenuated in mice lacking cGMP-dependent protein kinase G1 [77]. The NO donor, sodium nitroprusside (SNP), reduced the frequency of slow waves in rabbit USM [32] indicating that NO exerts its inhibitory effects in USM by inhibiting spontaneous activity. There is evidence that spontaneous activity in both UICs and USMCs is affected by NO, however, the degree to which these effects account for neurogenic relaxations

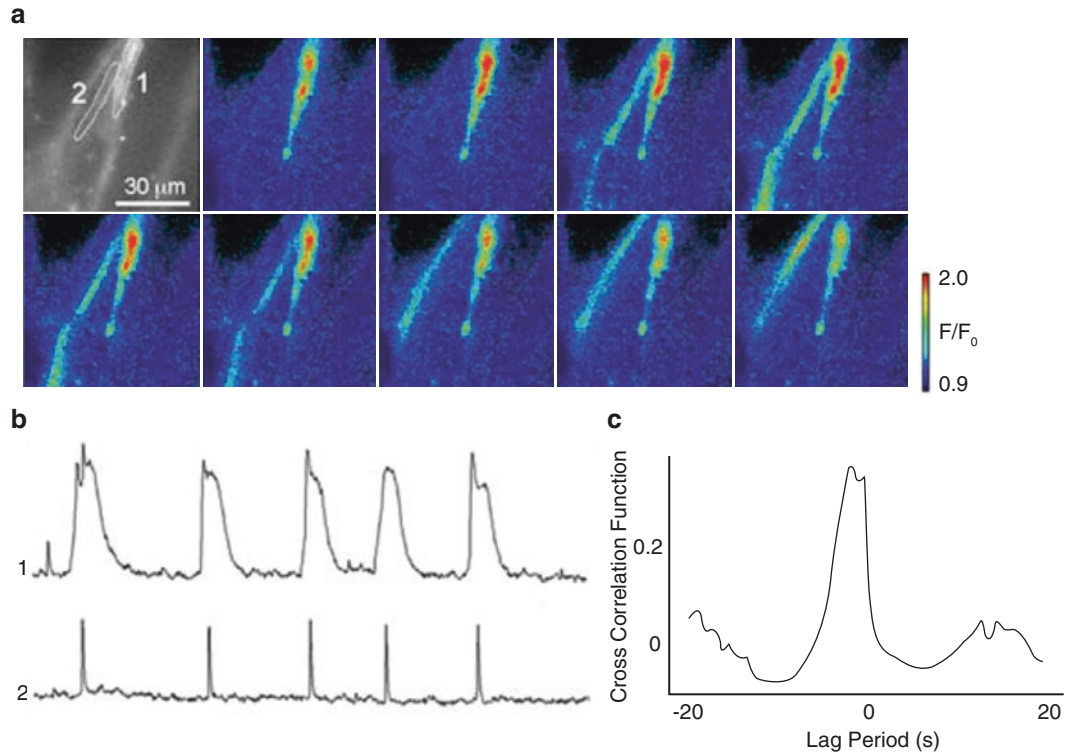

Fig. 6.8 Analysis of the temporal relationship of Ca^{2+} transients between UICs and USMCs. (**a**) A series of frames at intervals of 0.2 s demonstrating USMC Ca^{2+} transients originating from an UIC. (**b**) In the same preparation, synchronous Ca^{2+} transients were generated by an UIC (1) and a USMC (2). (**c**) A cross-correlogram for UICs and USMCs showed a peak near lag period zero. Adapted from [27]

of USM is still under debate. Application of the NO donor, DEA-NONOate, dramatically reduced the frequency of Ca^{2+} transients in USMCs, in situ [34] and relaxations induced by DEA-NONOate were absent in smooth muscle guanylyl cyclase knockout (SM-GCKO) mice USM [78]. These findings prompted the authors to conclude that 'NO-GC found in SMCs of the urethral sphincter mediates NO-induced relaxation'. Unfortunately, this study did not examine nerve-mediated NO responses, as responses to exogenous application of agonists and NO donors can often be quite different to those induced by stimulation of intrinsic nerves. For example, intramuscular ICC (IC-IM) in the murine fundus are known to mediate neural responses to NO, but W/W_v mice, which lack IC-IM can still relax in response to the NO-donor SNP [51]. Lies et al. also showed that DEA-NONOate-induced relaxations were maintained in mice lacking NO-guanylyl cyclase (NO-GC) in c-kit-positive ICC [78]. Therefore, if UICs are involved in mediating NO responses in the murine urethra, they are not c-kit positive.

Several lines of evidence indicate that UICs could be involved in NO signalling in the urethra. Treatment of USM with SNP led to increased levels of cGMP fluorescence in interstitial cells [50, 79] and stimulation of nitrergic nerves in USM, led to increased cGMP production in both UICs and SMCs [80]. Rabbit UICs (identified by c-kit immunoreactivity) had frequent points of contact with neuronal nitric oxide synthase containing nerves, consistent with the idea that they play a role in the inhibitory nitrergic neurotransmission of USM [81]. Sergeant et al. provided direct evidence that spontaneous activity in UICs was modulated by NO, as STDs, recorded under current clamp, and STICs recorded under voltage clamp in freshly isolated rabbit UICs were

inhibited by the NO donor, DEA-NO, the soluble guanylate cyclase activator, YC-1, or the cGMP analogue, 8-Br-cGMP [82]. Finally, spontaneous Ca^{2+} transients recorded in situ from UICs of the rabbit urethra, were inhibited by the NO donor, SIN-1 [27]. It appears that inhibitory effects of NO in UICs are mediated by activation of PKG, and not protein kinase A (PKA), as the PKG activator SP-8-br-cGMP reduced the frequency of STDs and Ca^{2+} waves, whereas application of the adenylate cyclase activator, forskolin or the membrane permeable cyclic AMP analogue, 8-Br-cAMP did not affect spontaneous Ca^{2+} waves in rabbit UIC [83].

6.5.2 Adenosine Triphosphate

Nitric Oxide is not the only inhibitory neurotransmitter in the urethra. Several studies have demonstrated that adenosine triphosphate (ATP) or its derivatives, adenosine and adenosine diphosphate (ADP), may also be potent inhibitory neurotransmitters in USM, since EFS of USM strips induced relaxations that were inhibited by purinergic receptor antagonists and mimicked by exogenous application of ATP [18–21]. However, it has also been noted that ATP, or related compounds, can produce excitatory effects in USM [21, 30, 84–86]. One explanation that could account for these opposite effects is the level of tone on the tissue prior to ATP application. For example, relaxant effects were observed in preparations which had been 'pre-contracted' with agonists such as arginine vasopressin or noradrenaline, whereas contractions were observed if ATP was applied at resting tone. Bradley et al. showed that the contractile effects induced by application of ATP to the rabbit urethra were mimicked by the P2Y receptor agonist, 2-MeSADP and were inhibited by the selective P2Y1 receptor antagonist, MRS2500 suggesting that P2Y receptors were involved in the excitatory effects of ATP in rabbit USM [85]. Involvement of UICs in this response was inferred from experiments which showed that ATP and P2Y receptor agonists increased the frequency of spontaneous Ca^{2+} waves and STICs in freshly isolated UICs (Fig. 6.9). Interestingly however, stimulation of purinergic nerves in strips of rabbit USM yielded contractions that were inhibited by desensitisation of P2X receptors using α,β-methylene ATP but were unaffected by the P2Y receptor antagonist MRS2500 [86]. This suggested that, in USM, neurally released ATP targeted P2X receptors and not P2Y receptors. Further investigation revealed that isolated rabbit USMCs had robust P2X receptor currents that could be inhibited by α,β-methylene ATP. Therefore, it appears that P2Y receptor-dependent responses in UICs are unlikely to be involved in purinergic nerve-mediated responses in rabbit USM.

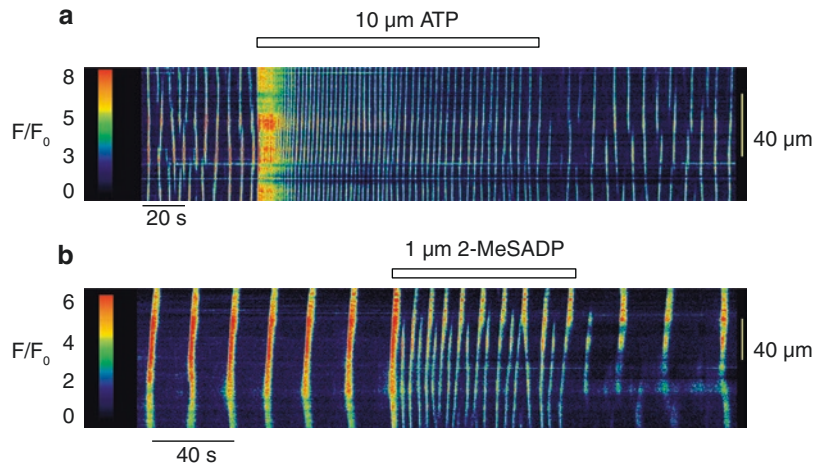

Fig. 6.9 Representative pseudo linescan images showing effects of ATP (**a**) and the P2Y1 receptor agonist 2-MeSADP (**b**) on Ca^{2+} waves in isolated rabbit UICs. Adapted from [85]

6.5.3 Noradrenaline

Noradrenaline (NA) is the principal excitatory neurotransmitter in the urethra [16, 17] and augments the level of urethral tone via activation of post-junctional α_1-adrenoceptors [13, 17]. Hashitani et al. indicated that α_1-adrenoceptor-mediated increases in tone resulted from up-regulation of the endogenous pacemaker mechanism, since exogenous application of NA increased the frequency of slow waves in rabbit USM [32]. Sergeant et al. demonstrated that spontaneous activity in freshly isolated UICs was also enhanced by NA suggesting that these cells could be involved in this response [25, 87]. The effects of NA were attenuated by CPA and 2-APB as well as niflumic acid and A-9-C, suggesting that activation of α_1-adrenoceptors led to release of Ca^{2+} from IP_3-sensitive stores, which in turn stimulated CACC. Similarly, bath application of the α_1-adrenoceptor agonist, phenylephrine increased the frequency of Ca^{2+} transients in UICs in situ and elevated intracellular Ca^{2+} levels [27]. Sergeant et al. [87] and Fedigan et al. [56] demonstrated that EFS-induced contractions of rabbit USM were inhibited by the CACC inhibitors, niflumic acid, A-9-C and $T16A_{inh}$-A01, respectively. Since CACC are more prominent in rabbit urethral UICs, compared to USMCs, it is tempting to speculate on a role for UIC in mediating neural responses in USM, as is the case for IC-IM in the gastric fundus and ICC in the deep muscular plexus (IC-DMP) of the small intestine [88]. However, a comparison of neural responses in USM lacking UICs has yet to be made and therefore this idea remains speculative. Furthermore, Ca^{2+} waves in murine SMC were also accelerated by application of phenylephrine indicating that USMCs can be directly modulated by NA [34]. Kyle et al. also showed that phenylephrine reduced depolarisation-evoked large conductance Ca^{2+}-activated K^+ (BK) currents in rabbit USMCs and suggested that the reduction in this current altered compound action potentials to promote excitability of USM [89].

Walsh et al. [90] demonstrated that contractile responses of rabbit USM in response to EFS and exogenous application of phenylephrine were all potently inhibited by the Rho-associated kinase (ROK) inhibitors, Y27632 and H1152 [90]. Surprisingly, however, ROK inhibition had no effect on the phosphorylation of the known ROK substrates, myosin regulatory light chains (LC20) at S19 or the myosin-targeting subunit of myosin light chain phosphatase (MYPT1), in either the absence or presence of contractile stimuli. Therefore ROK plays an important role in USM contraction induced by NA; however, the mechanisms underlying this response require further investigation.

6.5.4 Acetylcholine

Although the bladder neck and proximal urethra receive a rich cholinergic innervation [84], the functional role of acetylcholine (ACh) in the urethra is still unclear. Studies have indicated that inhibition of muscarinic receptors has little effect on urethral tone in vivo [91] or in vitro [14, 87]. However, stimulation of cholinergic nerves in vitro has been demonstrated to contract the urethra of sheep, pigs, dogs and rabbits [14, 22–24] and these effects appear to be mediated post-junctionally via activation of M_2 and/or M_3 muscarinic receptors [92, 93]. In the isolated sheep urethra, a significant component of the neurogenic contraction is sensitive to atropine [14] consistent with the idea that muscarinic stimulation can augment urethral tone. Similarly, application of cholinergic agonists to porcine urethra in vitro contracts the longitudinal and circular muscle layers equally. However, in the rabbit urethra, although an atropine-sensitive component of excitatory junction potentials has been demonstrated [24, 94], it was small compared to the α_1-adrenoceptor-mediated component, suggesting that muscarinic receptors contributed minimally to the electrical response to nerve stimulation in this preparation. In humans, there is little evidence that muscarinic receptor activation has any significant effect on intraurethral pressure in vivo [95].

In addition to the species variability noted above, a number of observations suggest that the response to muscarinic stimulation varies along the length of the urethra. Thus, Nagahama et al. dem-

onstrated that application of carbachol produced robust contractions in the male proximal urethra but had little effect on the distal urethra [96]. Interestingly, application of carbachol to strips of distal rabbit urethra pre-contracted with noradrenaline, resulted in relaxations of tone. These effects were abolished when NO synthase was inhibited, suggesting that muscarinic stimulation may also induce release of NO from nitrergic nerves.

The role, if any, of interstitial cells in cholinergic innervation of the urethra has not yet been ascertained. However, the cholinergic agonist carbachol evoked a series of oscillatory inward currents when applied to single UIC voltage clamped at −60 mV [26] and these events were inhibited by the CACC inhibitor A-9-C, suggesting a common activation pathway to that described by noradrenaline above. In contrast, Kyle et al. were unable to detect any inward currents elicited by muscarinic agonists in freshly dispersed rabbit USMCs [89]. However, they demonstrated that carbachol altered compound action potential characteristics, elicited by 1 s current injections. Therefore, it is apparent that electrical activity in both UICs and USMCs can be directly modulated by carbachol, but more experiments are required to elucidate the mechanisms involved in cholinergic responses at the whole tissue level.

6.6 Summary

Urethral smooth muscle generates spontaneous tone that makes a contribution to the maintenance of urinary continence by generating a urethral closure pressure that exceeds intravesical bladder pressure. Stress urinary incontinence (SUI) is common in women after vaginal delivery child birth [6] and is typically thought of as being due to reduced urethral support, referred to as urethral hypermobility. However, urethral hypermobility is not predictive of SUI and it is also recognised that it can coexist with a defective closure mechanism, known as 'intrinsic sphincter deficiency' [8, 97]. There are currently no FDA-approved pharmacological treatments for SUI, therefore it is crucial that the mechanisms responsible for the maintenance of urethral tone are elucidated, in order to identify novel therapeutic targets. This chapter provides a summary of the mechanisms responsible for spontaneous activity in urethral smooth muscle.

The urethra is regarded as a 'tonic' smooth muscle but, counterintuitively, there is now consensus that urethral smooth muscle tone is achieved by the averaging effect of numerous small asynchronous 'phasic' contractions in the tissue [27, 34, 73]. The phasic contractions of urethral smooth muscle appear to arise from activation of CACC/Ano1 channels and may originate in specialised cells referred to as urethral interstitial cells. This requires further investigation. Spontaneous activity in both USMCs and UICs involves Ca^{2+} release from intracellular stores and Ca^{2+} influx across the plasma membrane and is regulated by several autonomic neurotransmitters, including noradrenaline, acetylcholine, nitric oxide and adenosine triphosphate. Much of the work described in this chapter is based on animal studies, however it is apparent that there are species differences regarding the role of Ca^{2+} influx via L-type Ca^{2+} channels and on the cellular basis of Ano1 expression and activity. Therefore, it is imperative that future studies should examine the mechanisms responsible for spontaneous activity in human USM samples and examine if this activity, or its modulation by neurotransmitters, is altered in conditions that are associated with USM dysfunction, such as stress urinary incontinence.

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