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Modulation of lower urinary tract smooth muscle contraction and relaxation by the urothelium

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ABSTRACT

The epithelial inner layer of the lower urinary tract, i.e. the urothelium, and other parts of the mucosa are not just a passive barrier but play an active role in the sensing of stretching, neurotransmitters, paracrine mediators, hormones and growth factors and of changes in the extracellular environment. We review the molecular and cellular mechanisms enabling the urothelium to sense such inputs and how this leads to modulation of smooth muscle contraction and relaxation. The urothelium releases various mediators including ATP, acetylcholine, prostaglandins, nitric oxide and nerve growth factor. These may affect function and growth of smooth muscle cells and afferent nerves. However, the molecular identity of the urothelium-derived mediator directly modulating contractile and relaxant responses of isolated bladder strips has remained elusive. The morphology and function of the urothelium undergo changes with ageing and in many pathophysiological conditions. Therefore, the urothelium may contribute to the therapeutic effects of established drugs to treat lower urinary tract dysfunction and may also serve as a target for novel therapeutics. However, therapeutics may also change urothelial function, and it is not always easy to determine whether such changes are part of the therapeutic response or reflect secondary alterations.

Key words: urothelium, ATP release, muscarinic receptor; β -adrenoceptor; contraction; relaxation

List of abbreviations:

Ach, acetylcholine

CBS, cystathionine- β -synthase

CSE, cystathionine- γ -lyase

EFS, electrical field stimulation

H₂S, hydrogen sulphide

LUT, lower urinary tract

NGF, nerve growth factor

NO, nitric oxide

NOS, NO synthase

OAB, overactive bladder syndrome

PG, prostaglandin

TRP, transient receptor potential channel

UDIF, urothelium-derived inhibitor factor

Introduction

The urothelium is the epithelial inner layer of the hollow organs of the lower urinary tract (LUT). While it has long been considered as a passive barrier only, we now know that it is anything but. Firstly, it is much less of a barrier than anticipated and several xenobiotics can be absorbed upon intravesical installation to reach systemically relevant concentrations even in healthy animals (Krege et al., 2004). Second, the urothelium can become leaky under pathophysiological conditions (Parsons, 2011). Third, and most importantly, the urothelium is a highly active cell layer, which senses external stimuli, releases various mediators and thereby modifies smooth muscle tone and the function of other cell types in the bladder wall. We here review the role of the urothelium as a sensor, its modulation of LUT smooth muscle tone, the mediators involved in such modulation and the regulation of urothelial function in disease and upon treatment. We explicitly wish to refer readers to excellent previous reviews of various aspects of urothelial function (Apodaca et al., 2007, Birder and De Groat, 2007, Birder and Andersson, 2013, Andersson and McCloskey, 2014).

Methodological considerations

The urothelium is part of the mucosa, which also includes the basal membrane and the lamina propria. The latter is a complex structure including afferent nerve endings, interstitial cells similar to those of Cajal in the gut, blood and lymphatic vessels and, in some species including humans, a muscularis mucosae (Andersson and McCloskey, 2014, Fry and Vahabi, 2016). Although the urothelium is only part of the mucosa, some authors have used the terms ‘urothelium’ and ‘mucosa’ as synonyms, and it is not fully clear from reading the respective papers what exactly has been studied. Specifically, ‘urothelium removal’ (for instance to study

the role of urothelium-derived mediators on contraction and relaxation) appears to reflect removal of the entire mucosa in many cases. Possibly inappropriate use of terms should be considered when interpreting published data on the role of the urothelium in particular and of the mucosa in general. A summary of key advantages and limitations of various ways to study urothelial function and role is given in Table 1.

Studies on the function of the urothelium rely on a range of approaches, each of them having specific advantages and limitations. For instance, *in vitro* studies with bladder strips have often applied direct comparisons of strips after removal of the urothelium or entire mucosa with intact strips (see sections on modulation of contractile and relaxant responses). Differences between the two preparations point to the urothelium or mucosa as being involved; however, it does not always become clear whether the urothelial part is in the sensing of the signal and/or the response to it. As there are no approaches to remove the urothelium *in vivo*, conclusions about its function in a living animal or patient are difficult. A possible alternative, at least in experimental animals, are studies in which a gene of interest is knocked-out or overexpressed selectively in the urothelium (Schnegelsberg et al., 2010).

Given these limitations, some investigators have turned to freshly prepared urothelial cells, primary cultures thereof or to urothelium-derived cell lines. Such cell lines can be immortalized such as the non-malignant cell lines UROtsa or TERT-NHUC cells but can also originate from urothelial cancer such as J82 cells. However, the quantitative and qualitative expression pattern of urothelial cells may change in culture, and such changes may be even more extensive in urothelium-derived cell lines (Tyagi et al., 2006), and even more so in cell lines derived from urothelial cancer. Both conclusions were substantiated by later studies exploring a greater variety of receptors and other signaling-related molecules (Ochodnický et al., 2012, Bahadory et al., 2013). As the main limitation of freshly prepared urothelial cells is the availability of

large quantities, various attempts have been made with rat (Kurzrock et al., 2005) and human urothelial cells (Daher et al., 2004) to improve culturing methods, for instance by using matrices or organ-like culture. Nonetheless, the interpretation of data from cultured urothelial cells or urothelium-derived cell lines needs to consider phenotypic changes, which may limit the translational value of these models.

Other limitations exist that are not specific for research on the urothelium. The presence of receptors, channels and other molecules can be assessed and quantified at the mRNA level with relative ease; however, it remains unclear for most genes how predictive mRNA levels are for those of functional protein. Detection at the protein level has been based on radioligand binding in some studies on muscarinic receptors (Mansfield et al., 2005, Braverman et al., 2007, Anisuzzaman et al., 2008, Mansfield et al., 2009) but has relied additionally (for muscarinic receptors) and predominantly (for other receptors) on antibodies. Of note, the vast majority of antibodies against G protein-coupled receptors and ligand-gated ion channels lacks useful target specificity (Michel et al., 2009) but some carefully validated receptor antibodies are emerging (see below). While radioligands allow a more definitive detection of receptors, the ligands used for discrimination of subtypes typically had only moderate selectivity. Thus, either method has limitations that need to be considered.

Finally, the presence of a given protein or other molecule does not necessarily demonstrate that it is involved in a given functional response. Dissection of pathways leading to functional responses requires use of activators or, even better, inhibitors of specific steps leading from sensing of signal to cellular response. Such dissection can be done using genetic tools, such as siRNA, antibodies or small molecules; again, each of these tools comes with its own limitations as extensively discussed elsewhere (Michel and Seifert, 2015).

Urothelium as sensor

The physiological amount of urine in the bladder immediately after a void is close to 0 ml in humans but may be higher in species using urine for territorial marking behavior such as dogs. On the other hand, the healthy human bladder can store about 500 ml, which means that it undergoes major stretching during the micturition cycle. This requires sensing mechanisms that inform the brain about the filling state of the bladder and allow it to decide whether to continue storing or to void. The urothelium appears to play a central role in such sensing. It is also involved in the sensing of thermal and chemical stimuli (Birder and Andersson, 2013). Such sensing involves various ion channels and receptors and leads to the release of mediators such as ATP, acetylcholine (ACh) and nitric oxide (NO, see below). For instance, stretching can stimulate the release of mediators including ACh (Yoshida et al., 2006) and ATP (Ochodnický et al., 2013) from the urothelium. Stretch-induced release can further be augmented by mediators such as bradykinin (Ochodnický et al., 2013).

Urothelial cells express a range of cation channels. These include the transient receptor potential channels (TRP) TRPV1 and TRPV4. Expression of TRPV1 protein was reportedly greater in the superficial than in the basal cells, and much less expression was found in malignant tissue (Lazzeri et al., 2005). In contrast, TRPV4 was preferentially found close to adherence junctions, with similar expression in healthy and malignant human tissue and in healthy mice (Janssen et al., 2011). Such TRP channels are likely to be involved in the sensing of stretching and some pathophysiological conditions of the bladder (Nilius and Szallasi, 2014). The urinary bladder also expresses several big, small and intermediate conductance Ca^{2+} -activated K^{+} channels (Chen et al., 2004), but at least for the SK2 channel expression appears to exist at a lower level in urothelium as compared to bladder smooth muscle (Thorneloe et al., 2008). A difference in

contractile responses in the presence of the K⁺ channel opener rimalkalim in the absence and presence of urothelium in mouse, pig and human bladder strips (Wuest et al., 2005), has been taken as indirect evidence for a functional role of K⁺ channels in the urothelium (although these experiments most likely reflect a role in the mucosa in general). In line with this caveat, direct evidence for a functional role of KCNQ channels in the interstitial cells of the lamina propria has been obtained in guinea pigs (Anderson et al., 2009).

The urothelium does not only sense filling of the bladder, but can also sense presence of surrounding neurotransmitters, paracrine mediators and hormones. The primary physiological transmitter of the parasympathetic nerves innervating the human bladder is Ach (ATP may be a primary physiological parasympathetic transmitter in several non-human mammals; (Burnstock, 2014)). Moreover, Ach can be formed non-neuronally in the urothelium (Yoshida et al., 2006). Some studies have demonstrated expression of some subtypes of nicotinic Ach receptors at the mRNA level. In mice, $\alpha 2$, $\alpha 4$, $\alpha 5$, $\alpha 6$, $\alpha 7$, $\alpha 9$ and $\alpha 10$, but not $\alpha 3$ subunits were detected; while $\alpha 9$ was found only in the umbrella cells, $\alpha 4$, $\alpha 7$ and $\alpha 10$ subunits were also seen in the intermediate and basal cell layers (Zarghooni et al., 2007). The same group reported that the $\alpha 7$ subunit was much more abundant than the $\alpha 9$ and $\alpha 10$ subunits in human urothelium (Bschleipfer et al., 2007). However, most studies have explored the presence of muscarinic Ach receptors in the urothelium. Muscarinic receptors have been detected in the urothelium of every mammalian species investigated, including mouse (Zarghooni et al., 2007), rat (Cheng et al., 2007, Anisuzzaman et al., 2008), guinea pig (Grol et al., 2009), pig (Bahadory et al., 2013) and man (Mansfield et al., 2005, Tyagi et al., 2006, Braverman et al., 2007, Bschleipfer et al., 2007, Mansfield et al., 2009, Ochodnický et al., 2012). Studies in rat, pig and human have consistently reported a lower expression of muscarinic receptors in urothelium than in detrusor (Mansfield et al., 2005, Tyagi et al., 2006, Braverman et al., 2007, Cheng et al., 2007, Anisuzzaman et al., 2008, Bahadory et al., 2013); the sub-urothelial layer also stained positive for muscarinic

receptors, but other parts of the lamina propria did not (Grol et al., 2009, Bahadory et al., 2013). The most abundantly expressed subtypes at the mRNA and protein level appear to be M₂ and M₃ receptors (Braverman et al., 2007, Bschiepfer et al., 2007, Anisuzzaman et al., 2008, Ochodnický et al., 2012), but some subtypes may exhibit a rather restricted expression with M₁ restricted to basal cells, M₂ largely to umbrella cells, M₃ and M₄ homogeneously distributed and M₅ exhibiting a decreasing expression from luminal to basal (Bschiepfer et al., 2007). However, most of these findings were generated with antibodies shown to lack proper target selectivity (Jositsch et al., 2009). As most clinically used muscarinic antagonists exhibit moderate selectivity for one subtype at best, the clinical relevance of differential expression of muscarinic receptor subtypes within the mucosa remains to be elucidated.

Adrenoceptors are also expressed and functionally active in the urothelium. β -Adrenoceptor subtype mRNA is present in the urothelium of rats (Kullmann et al., 2011) and humans (Otsuka et al., 2008, Tyagi et al., 2009a, Ochodnický et al., 2012), and in human urothelium-derived cell lines (Harmon et al., 2005). The studies providing quantitative evaluation suggest that the β_2 -adrenoceptor subtype is expressed most prominently in both species (Kullmann et al., 2011, Ochodnický et al., 2012). Expression of all three β -adrenoceptor subtypes at the protein level has been reported based on immunohistochemistry in humans (Otsuka et al., 2008, Kullmann et al., 2011, Otsuka et al., 2013), but only some of these studies have applied validated antibodies. However, it is notable that the staining was more intense in urothelium than in smooth muscle in all of these studies, which is opposite to the expression pattern of muscarinic receptors (see above). One study reported that interstitial cells exhibit less expression than urothelium, but more than bladder smooth muscle (Otsuka et al., 2013). α_1 -Adrenoceptors have low abundance in the overall human or porcine bladder (Goepel et al., 1997), and *in situ* hybridization experiments did not detect α_{1A} -adrenoceptor subtype mRNA in human urothelium (Walden et al., 1997); the same study however, reported a high density in the

urothelium of rat and monkey bladder. An expression profiling study in freshly prepared human urothelial cells reported lack of mRNA for α_{1B} -, moderate amounts of α_{1A} - and strongest abundance of α_{1D} -adrenoceptors (Ochodnický et al., 2012). In contrast, the same study detected all three subtypes of α_2 -adrenoceptors. α_{1D} -Adrenoceptors were also detected by immunoblots in rat urothelium (Ishihama et al., 2006), albeit based on a non-validated antibody.

Presence of a wide range of other receptors has been explored in isolated studies. For instance, freshly isolated human urothelial cells expressed mRNA for serotonin 5HT_{2A} but not 5HT_{1B} receptors, angiotensin II type 1 but not type 2 receptors, high levels of endothelin ET_A and ET_B receptors and of all five subtypes of sphingosine-1-phosphate receptors as well as the sphingosine kinases 1 and 2 generating their ligand based on array technology (Ochodnický et al., 2012) but not verified by PCR based on validated primers. Bradykinin B₁ and B₂ receptor mRNA was found in human urothelial cells (Ochodnický et al., 2012); in contrast, only B₂ mRNA was present in normal rat urothelium but B₁ receptors became detectable in cyclophosphamide-induced cystitis (Chopra et al., 2005). Another study at the whole bladder level, found up-regulation of B₁ receptor mRNA upon spinal cord injury; immunohistochemical experiments showed an increased expression of B₁ receptor protein in the urothelium (Forner et al., 2012), but this was based on antibodies without proper validation. Freshly isolated human urothelial cells expressed mRNA for substance P (NK1), substance K (NK2) and neuromedin K receptors (Ochodnický et al., 2012). Pig bladder has a similar expression of substance K receptor mRNA in smooth muscle and suburothelium with less but detectable expression in the urothelium (Bahadory et al., 2013). P2Y₆ receptor mRNA is also found in the urothelium but at lower abundance than in suburothelium and smooth muscle, whereas P2X₁ mRNA is only present in suburothelium and smooth muscle and not in urothelium. While prostaglandin EP1 receptors exhibited only weak staining in urothelium and suburothelium of the guinea pig bladder, strong staining of EP2 receptors was found in both layers, albeit on limited validation

of the antibodies being used (Rahnama'i et al., 2010). Cannabinoid CB1 and CB2 receptor mRNA was found in human urothelium, possibly with higher expression than in smooth muscle (Tyagi et al., 2009b). The expression level of CB1 appeared higher than that of CB2, and this was confirmed in a study of freshly isolated human urothelial cells (Ochodnický et al., 2012).

Nerve growth factor (NGF) is a key modulator of bladder function (Ochodnický et al., 2011). Urothelium produces and releases NGF (see below) and expresses receptors for it. Freshly isolated human urothelial cells expressed mRNA for its low-affinity receptor p⁷⁵ and, to a lesser extent, for the high-affinity receptor trkA (Ochodnický et al., 2012). Immunohistochemical studies confirmed strong expression of p⁷⁵ in human urothelium (Vaidyanathan et al., 1998, Eryildirim et al., 2006), albeit with poorly validated antibodies.

Modulation of contractile responses

Alongside the sensory functions, it has become clear that the urothelium has a local and direct influence on the contractility of the underlying smooth muscle in the lower urinary tract. This modulatory action was initially demonstrated as an inhibitory effect on bladder detrusor contractility, and was first reported in the guinea pig bladder, in which substance P-induced contractions were enhanced in mucosa-free bladder strips compared to their mucosa-intact counterparts (Maggi et al., 1987). Thereafter, this inhibitory effect of the urothelium on detrusor smooth muscle contraction has been reported in bladders from many species, including mouse (Wuest et al., 2005, Meng et al., 2008, Canda et al., 2009), guinea pig (Guan et al., 2014), cat (Levin et al.), dog (Saban et al., 1992), pig (Hawthorn et al., 2000, Buckner et al., 2002, Sadananda et al., 2008) and human (Chaiyaprasithi et al., 2003, Wuest et al., 2005, Propping et al., 2013). In 2000, Hawthorn et al., confirmed, using a bioassay experiment in which a

urothelium-intact strip was co-incubated with a denuded strip, that the inhibitory effect was due to a diffusible urothelium-derived inhibitory factor (UDIF) which was released from the pig bladder urothelium (Hawthorn et al., 2000). The effect observed is an inhibition of contractions rather than a direct relaxation. Although unidentified in nature, UDIF was released in response to muscarinic stimulation, but not in response to elevated extracellular KCl or neurokinin A (Hawthorn et al., 2000). Muscarinic-receptor mediated release of the inhibitory factor is consistent across most species, apart from dog (Saban et al., 1992). However, an inhibitory effect of the urothelium has also been demonstrated following stimulation with KCl in the mouse (Wuest et al., 2005), cat (Levin et al.) and human bladder (Wuest et al., 2005, Propping et al., 2013), in response to ATP in the cat bladder (Levin et al.), and in response to neurokinin A in the dog bladder (Saban et al., 1992) and following nerve-stimulation in cat (Levin et al.) and human bladder (Chaiyaprasithi et al., 2003). Thus, the stimulus for release may be species dependent. Moreover, a species-specific controversy appears to exist in the rat bladder with respect to UDIF. Some studies have shown that the urothelium does not appear to have an inhibitory effect on smooth muscle contractility in the rat bladder (Pinna et al., 1992), whilst others have used a gentle swabbing technique to remove only the urothelial layer and found an inhibitory effect (Munoz et al., 2010).

Although some early reports demonstrated a non-adrenergic, non-nitroergic, non-prostanoid relaxation factor in the rat bladder, this was shown to be urothelium-independent (Fovaeus et al., 1999, Bozkurt and Sahin-Erdemli, 2004). Adding to the complexity, the rat bladder the urothelium releases prostaglandins that excite and contract the bladder, rather than inhibit contractions (Nakahara et al., 2003).

Most studies of the inhibitory effect of the bladder urothelium have been performed on the bladder dome, but the urothelium of the bladder trigone has a similar inhibitory effect on

smooth muscle contractility, and whilst release of the inhibitory factor is stimulated by muscarinic, and histamine, receptor activation in this region, it is not seen following stimulation of α_1 -adrenoceptors with phenylephrine (Templeman et al., 2002). However, complex cross-talk appears to occur between the receptor systems, since an inhibition of phenylephrine-mediated contractions is observed following muscarinic or histamine receptor stimulation.

In addition to the large voiding contractions evoked by motor nerves, which initiate bladder emptying, the bladder also demonstrates spontaneous contractile activity which is non-neuronal in origin. Whilst the role of this spontaneous activity in bladder function, and the cellular origin, is a focus of ongoing research, the urothelium can modulate spontaneous activity in a dramatic way. Removal of the urothelium from bladder wall preparations significantly reduced spontaneous contractions in the guinea pig (Sui et al., 2008) and rat bladder (Kanai et al., 2007). This is in complete contrast to the inhibitory action of the urothelium on tonic contractions described above. In contrast, in the mouse bladder the frequency of nifedipine-sensitive spontaneous activity of detrusor increased when the urothelium was removed (Meng et al., 2008) and a similar finding was reported in pig bladder (Buckner et al., 2002). The findings in pig bladder show that removal of the urothelium delays development of spontaneous contractions in the dome, although not in the trigone, but does not influence frequency or amplitude of spontaneous contractions (Akino et al., 2008). Thus, the modulatory effect of the urothelium on bladder spontaneous contractility is still a matter of debate. Either way, whilst still far from being understood, it has been proposed that the urothelium may have a dual effect on contractility, an inhibitory influence on tonic contractions via a diffusible agent and an excitatory influence on spontaneous contractility via direct cellular interaction with underlying cells, potentially the interstitial cells in the lamina propria (Birder et al., 2010, Fry and Vahabi, 2016).

It is easy to see how the inhibitory influence of the urothelium on bladder tonic contractility may be important for normal bladder compliance, since during filling intravesical pressure remains low until the initiation of tonic contractions and voiding. With respect to the stimulatory action on spontaneous activity, this may reflect a mechanism whereby the bladder is maintained at its smallest volume during filling, to optimize sensation of bladder filling.

Whilst the urothelium lines the urinary tract from the renal pelvis through to the upper urethra and even the glandular portions of the prostate, there has been limited focus and much less is known about the influence of the urothelium on the smooth muscle of the non-bladder regions of the LUT. In the urethra, the urothelium changes from a transitional epithelium to become stratified or columnar (Romih et al., 2005). However, an inhibitory influence of the urothelium on smooth muscle contractility has been demonstrated in the urethra, similar to that observed in the bladder. The urothelium of the urethra inhibits noradrenaline-induced contractions, but not cholinergic contractions in the hamster (Pinna et al., 1992), contractions to phenylephrine, high KCl and nerve-stimulation in the cat (Levin et al.), and bradykinin-induced contractions in the pig bladder neck (Ribeiro et al., 2014). We have recently shown that the pig urethra releases a diffusible inhibitory factor which reduces contractions to carbachol, noradrenaline and phenylephrine by similar amounts (Figure 1).

<<<insert Figure 1 approximately here>>>

Even less well researched is the urothelium of the ureter, although recent evidence suggests that it can influence the underlying smooth muscle in a manner similar to that seen in the bladder dome, trigone and urethra (Roedel et al., 2018). The urothelium in the human ureter can inhibit tonic, but not spontaneous or KCl-stimulated contractions. In the rat ureter the urothelium acts to prevent spontaneous contractile activity and also decrease potential excitatory effects of

endogenous contractile agents including carbachol, bradykinin and angiotensin II on ureteral motility (Mastrangelo and Iselin, 2007). This effect appears to be via the release of a relaxing agent rather than an inhibitory factor, although responses to NKA and vasopressin were enhanced by urothelium denudation. (Mastrangelo and Iselin, 2007).

Modulation of relaxant responses

In addition to the inhibitory actions on contractility of LUT smooth muscle described above, the urothelium is also able to modulate relaxation responses. Although fewer studies have investigated this effect, when compared to the number of studies on the inhibitory effects of the urothelium on contraction, the evidence has led to the proposal of the release of a urothelial-derived mediator that is excitatory. Alternatively, the effect may be explained by a direct excitatory cellular interaction of the urothelium with underlying cell types. Regardless of the underlying mechanism, the evidence highlights the multiple roles that the urothelium seems to play in smooth muscle modulation.

Relaxing effects of catecholamines are blunted by the presence of the urothelium in the human bladder (Otsuka et al., 2008, Propping et al., 2013, Propping et al., 2015b) and are mediated via β_2 -adrenoceptors (Propping et al., 2013). A similar effect was seen in mouse bladder, and the urothelium reduced the sensitivity of KCl pre-contracted tissues to β -adrenoceptor-induced relaxation to isoprenaline, again via β_2 -adrenoceptors, although at high concentrations of isoprenaline there was a minor involvement of β_3 -adrenoceptors (Propping et al., 2015a). Whilst in human bladder this inhibitory effect on relaxations was seen in tissues pre-contracted with KCl and carbachol (Propping et al., 2015b), the same authors showed in mouse bladder that the urothelium did not inhibit relaxations if the tissues were pre-contracted with carbachol

(Propping et al., 2015a). The rat bladder urothelium does not appear to influence smooth muscle relaxation to β_3 -adrenoceptor agonists (Kullmann et al., 2011). Interestingly, in the pig bladder, we have shown that whilst relaxation responses to isoprenaline are not modulated by the urothelium, the urothelium is involved in mediating inhibitory effects of isoprenaline on cholinergic detrusor contractions, with isoprenaline being more potent at inhibiting carbachol-induced contractions when the urothelium is present (Murakami et al., 2007, Masunaga et al., 2010). This effect was mediated by the β_3 -adrenoceptor (Masunaga et al., 2010).

Evidence is also conflicting with regard to the role of the urothelium in modulation of non-receptor mediated relaxations of the bladder smooth muscle. The urothelium does not modulate relaxations to the K_{ATP} channel opener rimalkalim in the mouse, pig or human bladder (Wuest et al., 2005). Although, in contrast, we have also shown that the urothelium does inhibit the relaxant effects of cromakalim on spontaneous contractions of the pig bladder dome, but not in the trigone region (Akino et al., 2008), suggesting that the mechanism for generation of spontaneous contractions in the smooth muscle involves modulation by the urothelium in the dome, though not in the trigone.

There is very limited information on the modulatory effect of the urothelium on relaxations of the non-bladder regions of the lower urinary tract. Prostaglandin (PG) E_2 and 2-methyl-thio-ATP-induced relaxations of hamster urethra do not seem to be modulated by the urothelium (Pinna et al., 1996). However, relaxation responses of hamster urethra to electrical field stimulation (EFS) are absent if the urothelium is present, demonstrating an inhibitory modulation of nerve-mediated relaxations, which is absent if L-NAME and suramin was present (Pinna et al., 1996). However, this appears to be species dependent, since EFS-induced relaxations were not affected by the presence of an intact urothelium in the cat urethra (Levin et al., 1995).

In conclusion, the urothelium acts as a sensor for changes in bladder volume, neurotransmitters, pH, temperature and various chemicals. In turn it influences smooth muscle activity, inhibiting muscle contraction to many stimuli and also inhibiting some relaxation responses of the detrusor. These effects of the urothelium on smooth muscle occur following the release of a number of mediators, some of which are well documented (e.g. ATP), whilst others have yet to be identified (e.g. UDIF).

Known mediators released from urothelium

The urothelium releases a wide variety of signalling molecules ranging from the classic neurotransmitter Ach to the more recently recognised gaseous transmitter hydrogen sulphide (H₂S). The release of some of these substances is well documented and their actions on other tissues such as sensory nerves well established. However, our knowledge of their actions on smooth muscle is limited. The actions of exogenously administered factors can be readily demonstrated in many cases, but very few studies have examined whether factors released from the urothelium reach the detrusor muscle at quantities large enough to modulate smooth muscle tone and contractions. Research in this field has increased enormously in recent years. However, the findings are complex, since many studies have been performed on bladder strips and bladder wall sections, as well as in whole bladders, which invariably contain additional non-urothelial cellular elements within the lamina propria or suburothelium. Thus, it is difficult to rule out mediator release from other cell types, but studies on isolated urothelial cells and cell lines are helping to clarify findings somewhat. Evidence for urothelial release and smooth muscle action will now be considered individually for each proposed mediator.

Adenosine 5-triphosphate (ATP)

Stretch-induced release: ATP release from the urothelium was first described by Fergusson et al. (Ferguson et al., 1997) and it is now the most researched signalling molecule released by the urothelium. Human urothelial cell lines, both of malignant (RT4 cells) and immortalised but non-malignant origin (UROtsa and TRT-HU1), spontaneously release ATP and this is greatly enhanced during cellular stretch most commonly induced by osmotic swelling in hypotonic solution (Sun and Chai, 2006, Birder et al., 2013, Mansfield and Hughes, 2014a, Mansfield and Hughes, 2014b, Kang et al., 2015, Sano et al., 2016, Farr et al., 2017). Similarly, findings have been reported for primary cultures of urothelial cells from the guinea pig (McLatchie and Fry, 2015), cat (Birder et al., 2003) and pig (Cheng et al., 2011b).

In isolated tissue strips, mechanical stretch induces release of ATP from guinea pig (Young et al., 2012, Sui et al., 2014), pig (Kumar et al., 2004, Sadananda et al., 2012, Smith et al., 2014, Kang et al., 2015) and human bladder (Kumar et al., 2007, Kumar et al., 2010, Sui et al., 2014, Sano et al., 2016), while mechanical force induces release in guinea pig urothelial cells (Kullmann et al., 2008b). *In vivo* release of ATP into the bladder lumen has been documented for the rat and human bladder (Jeremy et al., 1987, Timóteo et al., 2014). In isolated tissue studies, removal of the epithelium from rat bladder strips by swabbing prevents ATP release indicating that the urothelium is the sole source of ATP in this species (Munoz et al., 2010). In contrast, the release of non-neuronal ATP is not restricted to the urothelium in the pig bladder, but also occurs from interstitial cells in the lamina propria and also from the smooth muscle. However, the majority of ATP release during stretch still occurs from the urothelial cells (Cheng et al., 2011a). Thus, ATP may be released from non-neuronal, non-urothelial sources in the bladders of some species, but the predominant source still appears to be the urothelium.

Chemical-induced release: In addition to stretch, a number of drugs are known to induce ATP release including β -adrenoceptor agonists (Birder et al., 2002b), substance P (Munoz et al., 2010), pituitary adenylate cyclase activating polypeptide (PACAP) (Girard et al., 2008), TRPV1 agonists (Birder et al., 2002a) and also some cytokines such as epidermal growth factor and anti-proliferative factor (Sun et al., 2009). Muscarinic agonists also stimulate release of ATP, the response mediated via the M₂ receptor subtype, at least in the guinea pig (Munoz et al., 2010, Sui et al., 2014). Sui et al. (2014) found that ATP itself can exert an autocrine action, enhancing further ATP release via the activation of P2Y₆ receptors.

Mechanisms of ATP release: The release of classic transmitters from nerves involves the fusion of storage vesicles with the plasma membrane. However, the release of ATP from the urothelial cells appears to be more complex. A number of toxins that prevent the exocytotic release of transmitters have been used to address this question and the studies have become more clinically relevant as these toxins increasingly become available to treat the overactive bladder (Chancellor, 2017). Rat and human urothelial cells possess the protein target SNAP-23 and SNAP-25 for botulinum toxin, and treatment of urothelial cells with this toxin reduces the release of ATP and surprisingly increases release of NO (Hanna-Mitchell et al., 2015). The contrasting effects of the toxin on ATP and NO release confirmed a previous study in the rat, in which these changes in urothelial mediators were associated with changes in afferent nerve activity (Collins et al., 2013). These reports support the concept of vesicular release of ATP from urothelial cells, but botulinum toxin also exerts other effects such as a reduction in P2X₃ and TRPV1 receptors that may alter urothelial function (Apostolidis et al., 2006). Furthermore, evidence is mounting that other mechanisms may be operating in the urothelium. Recently pannexin channels, which have been demonstrated to have a role in the non-neuronal release of ATP in non-urinary tissues, have been identified in urothelial cells (Negoro et al., 2014). Inhibitors of these channels reduce ATP release in the rat bladder and depress release from

human urothelial cells, while mice lacking these channels also exhibit reduced ATP release from the urothelium (Negoro et al., 2014, Beckel et al., 2015, Silva et al., 2015). In anaesthetised rats, stimulation of P2Y₆ receptors causes release of urothelial ATP and increases voiding frequency, but inhibition of pannexin channels reverses both the change in ATP release and the bladder overactivity (Timóteo et al., 2014). Silva et al. (Silva et al., 2015) confirmed that this purinergic autocrine action mediated via P2X₆ receptors occurs via pannexin channels rather than vesicular release.

ATP release induced by stretch also involves pannexin channels with inhibitors of these channels reducing ATP release in rat and human urothelium (Negoro et al., 2014). Other channels have also been linked to ATP release and urothelial cells from mice lacking P2X₇ channel receptors (Negoro et al., 2014) or mechanosensitive Piezo channels (Miyamoto et al., 2014) exhibit reduced ATP release. Thus, ATP release appears to involve a number of mechanisms covering vesicular release and release via channels. It seems likely at this stage that both mechanisms operate to enable ATP release.

Functions of urothelial ATP: In recent years, growing evidence supports the role of urothelial derived ATP in mechanosensation. It is now generally accepted that bladder filling results in stretch of the urothelium stimulating the release of ATP, which acts on suburothelial sensory nerves to signal bladder fullness to the CNS. All aspects of this transduction system have been explored and confirmed: ATP is released during urothelial stretch, P2X₃ receptors are present on sensory nerves (Lee et al., 2000), stimulation of which activates bladder sensory nerves. Furthermore, bladder dysfunction has been observed in P2X_{2/3} gene knock out animals (Cockayne et al., 2000, Vlaskovska et al., 2001, Cockayne et al., 2005). Another autocrine action has also been reported where ATP via P2X receptors regulates endocytosis and

exocytosis to control urothelial surface area as the bladder stretches (Truschel et al., 2002, Wang et al., 2005).

However, it is less clear whether urothelium-derived ATP can directly influence smooth muscle contraction. Detrusor smooth muscle expresses P2X₁ purinoceptors, and exogenous ATP induces contraction of the smooth muscle in animals (Lee et al., 2000) and in human bladder (Harvey et al., 2002). However, evidence that endogenous ATP from the urothelium influences smooth muscle contraction is scarce, although in guinea pig bladder strips with an intact urothelium, carbachol enhances spontaneous contractile activity at low concentrations that have no effect when applied directly to the muscle (Sui et al., 2014). Since ATP is known to regulate this activity and carbachol induces release of ATP from the urothelium, the results suggest that low dose carbachol may induce contractile activity indirectly via urothelial ATP release. This conclusion is further supported by the finding that P2X receptor desensitisation also prevented the spontaneous activity (Sui et al., 2014).

Another mechanism by which urothelial ATP may influence detrusor activity is via suburothelial interstitial cells, which possess multiple purinergic receptor subtypes. In the human bladder both P2X₁ and P2X₃ receptors have been identified on the interstitial cells where they modulate cell spontaneous activity (Cheng et al., 2011b). These cells are coupled to each other and smooth muscle via gap junctions (Ikeda et al., 2007) and thus the initial signal of ATP release may be transported deep into the smooth muscle following a local effect on interstitial cells.

Thus, in conclusion, the release of ATP from the urothelium has been demonstrated in all species so far studied. The stimuli for release includes stretch, shear stress, cholinergic, adrenergic and tachykinin receptor stimulation. There is also a purinergic autocrine feedback

mechanism enhancing release. The function of ATP in mechanosensation is well established, but it also influences smooth muscle spontaneous contractile activity. Release of ATP into the urine of patients during bladder filling has been shown, although in this study ATP release appeared to occur early in filling with quite low bladder volumes (Jeremy et al., 1987, Cheng et al., 2014), suggesting ATP may have other roles to play in the bladder other than simply mechanotransduction.

Acetylcholine (ACh)

A non-neuronal cholinergic system exists within the urothelium, and Ach release has been widely demonstrated in mouse, pig, guinea pig and human bladder urothelium preparations (Lips et al., 2007, Nile and Gillespie, 2012, Smith et al., 2014), human bladder strips (Yoshida et al., 2006, Bschiepfer et al., 2012, Silva et al., 2015), as well as in isolated rat (Hanna-Mitchell et al., 2007), guinea pig (Kullmann et al., 2008b) and human urothelium cells (Kang et al., 2013, Li et al., 2013, Farr et al., 2017).

Whilst the urothelium can synthesize and release Ach, the mechanisms by which it does so are very different to those seen in cholinergic neurons. The presence of high-affinity choline transporter CHT1 and Ach-synthesizing enzyme choline acetyltransferase is controversial (Yoshida et al., 2006, Hanna-Mitchell et al., 2007, Yoshida et al., 2008, Bschiepfer et al., 2012), and it has been proposed that ACh is synthesized in the urothelium predominantly by the enzyme carnitine acetyltransferase (Lips et al., 2007). In terms of stimulus for release, both mechanical stress (McLatchie et al., 2014) and stretch of the urothelium (Yoshida et al., 2006, Hanna-Mitchell et al., 2007, Nile and Gillespie, 2012, Kang et al., 2013, Farr et al., 2017) can evoke Ach release, and, at least in cells, this seems to be an all-or-nothing event, rather than related to degree of stress (McLatchie et al., 2014). ATP is a potent chemical stimulus for Ach

release (Hanna-Mitchell et al., 2007, Stenqvist et al., 2017) and this is mediated partly by P2Y₆ receptors in the human bladder (Silva et al., 2015), and PGE₂ also stimulates Ach release (Nile and Gillespie, 2012).

The mechanism by which Ach is released from urothelial cells does not involve vesicular exocytosis (Hanna-Mitchell et al., 2007, Lips et al., 2007, Bschiepfer et al., 2012), and is independent of extracellular calcium, connexins and pannexins. Instead, the anion channel CFTR, intracellular calcium (Bschiepfer et al., 2012), and the organic cation transporters OCT1 and OCT3 play a role (Hanna-Mitchell et al., 2007, Lips et al., 2007, Bschiepfer et al., 2012).

Autocrine and paracrine ACh signaling occurs within the urothelium, although the precise role in normal bladder function is not yet clear. Ach can act via a negative feedback mechanism to inhibit further Ach release from the urothelium (Hanna-Mitchell et al., 2007) whilst, in contrast, muscarinic receptor agonists evoked Ach release from primary human bladder urothelial cells (Li et al., 2013). Adding to the complexity, Ach causes release of ATP from the urothelium (Kullmann et al., 2008a, McLatchie et al., 2014) and might also indirectly affect the release of NO, tachykinins and prostanoids (Hanna-Mitchell et al., 2007, Kullmann et al., 2008b, Nile and Gillespie, 2012). The question of whether urothelial Ach directly contracts bladder smooth muscle was recently partly addressed by Stenqvist et al. (2017), who showed that ATP stimulates release of urothelial Ach, which contributes to purinergic contractile responses of the rat bladder.

Whilst our understanding of non-neuronal Ach in the urothelium of the bladder has increased, Ach in the urothelium within the remainder of the LUT is a relatively untouched area of research. So far, the only evidence comes from a study of the urothelium of the rat urethra, which can release Ach from specialized polymodal urethral chemosensory cells (urethral brush

cells) in response to stimulation of bitter receptors and a cholinergic negative autocrine feedback mechanism for Ach release appears to exist in this tissue (Deckmann et al., 2018).

In conclusion, Ach is released from urothelial cells and the mechanism of choline uptake, Ach synthesis and Ach release differ from those involved in neuronal cholinergic systems. There is some evidence for urothelial Ach having actions on smooth muscle but this is limited and requires further study.

Nitric oxide (NO)

NO is a gaseous transmitter with inhibitory functions in most systems of the body. It is formed from L-arginine by NO synthases (NOS). These enzymes exist intracellularly as three different isoforms: two calcium-dependent constitutively expressed forms, endothelial NOS (eNOS) and neuronal NOS (nNOS) and one calcium-independent inducible form (iNOS), which is expressed under some conditions such as inflammation (Birder et al., 2005). The main form present in the healthy urothelium has been shown to be nNOS in several animal species including the rat (Birder et al., 2002b, Chuang et al., 2013), guinea pig (Gillespie et al., 2005) and cat (Theobald, 2003), while eNOS has been identified in the rat bladder (Giglio et al.). It has been suggested that eNOS is the only form present in the healthy human bladder (Fathian-Sabet et al., 2001), but others have found it in the urothelium and the interstitial cells of the lamina propria of the human bladder (De Ridder et al., 1999). Furthermore, iNOS is up-regulated in bladder cancer (Lin et al., 2003) and can be induced during inflammatory conditions such as interstitial cystitis (Birder et al., 2005, Andersson et al., 2012) and following treatment with lipopolysaccharide (Weng et al., 2009). A reduction in nNOS and simultaneous increase in iNOS in urothelial cells has been observed in an obstructed bladder outlet model in the rat (Johansson et al., 2002a). These changes in NOS are likely related to inflammation and

the release of cytokines that have been shown to enhance NOS expression (Johansson et al., 2002b).

A number of stimuli causing non-neuronal NO release has been identified and these include capsaicin, a TRPV1 receptor agonist which stimulates release from the mucosa and sensory nerves (Birder et al., 1998), β -adrenoceptor agonists (Birder et al., 2002b) and substance P (Munoz et al., 2010). The latter study also showed that the NO originated in the lamina propria rather than the urothelial cells.

The role of NO of neuronal origin in the bladder outlet is well established where it relaxes the smooth muscle during voiding, thus preventing any large rise in luminal pressure (Andersson and Persson, 1994). The role of NO of mucosal origin is difficult to determine due to the multiple sources of NO within the bladder including the urothelium and interstitial cells within the lamina propria (Munoz et al., 2010) and due to its multiple action that include modulation of afferent nerve function activity (Aizawa et al., 2011) and influences on smooth muscle and interstitial cell function (Gillespie et al., 2004). Surprisingly, detrusor muscle itself is not very sensitive to NO, and NOS inhibitors have little effect on the contractions of the detrusor smooth muscle induced by muscarinic agonists or nerve stimulation (Frazier et al., 2005). Inhibition of NO synthase enhances contraction amplitude in response to EFS (Garcia-Pascual et al., 1991), while NO donors induce detrusor smooth muscle relaxation in precontracted tissues (Hernández et al., 2008). However, the effect on detrusor smooth muscle is small compared to the effects of NO on vascular smooth muscle. Even when comparing the effect on detrusor to its actions on the bladder outlet region, the influence of NO on detrusor contraction is relatively minor (Kedia et al., 2009). Furthermore, in the normal rat bladder, NOS inhibition has little or no effect on detrusor contractile responses to muscarinic stimulation (Andersson et al., 2008, Andersson et al., 2012).

However, NO appears to play a far more significant role during inflammation. In the rat bladder during inflammatory responses induced by cyclophosphamide, the mucosa exerts a considerable (25%) inhibitory effect on detrusor contraction, and muscarinic responses are enhanced when the mucosa is removed. This inhibitory effect in this situation can be reversed in the presence of a NOS inhibitor (Andersson et al., 2008). Thus, it appears that muscarinic agonists induce a direct stimulatory effect on smooth muscle, which is partly reversed by NO release from the mucosa in inflammatory states.

The actions of NO are mediated via cGMP; the targets of NO action have been identified by examining cGMP immunoreactivity after stimulation of the bladder with NO donors and these include detrusor smooth muscle, urothelial and interstitial cells (Fathian-Sabet et al., 2001, Gillespie et al., 2006). It has been suggested that it is the interstitial cells within the suburothelium that are most sensitive to urothelial NO rather than the smooth muscle (Gillespie et al., 2005). These interstitial cells are thought to relay information from the urothelium to the smooth muscle and thus provide an indirect mechanism by which NO may influence smooth muscle contraction. Another indirect mechanism is via effects on smooth muscle growth, since NO donors have an inhibitory effect on the proliferation of smooth muscle cells (Johansson et al., 2002a).

Thus, NO appears to have only a minor direct role in regulating smooth muscle contraction but may influence muscle tone indirectly by modulating interstitial cells in the suburothelium, which in turn may influence smooth muscle tone. Since the actions of NO are mediated via cGMP and are inhibitory to cells, this indirect action may possibly explain the excitatory effects that are occasionally observed in bladder. Although the vast majority of reports concerning NO have indicated that it exerts inhibitory actions on smooth muscle, there is also some evidence

to suggest that NO can exert excitatory effects. In the isolated mouse bladder, NO can increase phasic contractile activity (Gillespie and Drake, 2004) and in pre-contracted human detrusor strips, both relaxation and contraction responses to NO donors have been observed (Moon, 2002).

In conclusion, the role of NO in the bladder is complex. It is released from multiple sites and has multiple targets of action. Furthermore, this complex role appears to change in disease states following the induction of iNOS.

Other gaseous transmitters

In addition to NO, there are two other gaseous transmitters, H₂S and carbon monoxide. Although there is currently no evidence for the urothelium releasing carbon monoxide, several studies have examined the H₂S systems in the bladder. Surprisingly, most of the studies have been performed on human tissues and cells. The human urothelium possesses the H₂S synthesising enzymes, cystathionine- β -synthase (CBS) and cystathionine- γ -lyase (CSE). These enzymes catalyse the conversion of L-cysteine to H₂S and both enzymes have been located to the urothelium of the human bladder dome (Fusco et al., 2012). Also, the H₂S precursor L-cysteine relaxes human bladder strips, an effect that is blocked by inhibitors of CBS and CSE (Fusco et al., 2012). Furthermore, in human cultured T24 urothelial cells, stimulation with carbachol causes the release of H₂S and again this is prevented in the presence of a CBS inhibitor. The release of H₂S induced by carbachol in human T24 urothelial cells was mediated via the activation of M₁ and M₃ muscarinic receptors (d'Emmanuele di Villa Bianca et al., 2016). It is still early days in the study of H₂S, but the functions of H₂S in the bladder appears two-fold: an autocrine effect on the urothelium and an inhibitory effect on the detrusor muscle. H₂S appears to exert an autocrine effect by elevating intracellular cGMP levels in urothelial

cells, the consequences of which are yet to be established (d'Emmanuele di Villa Bianca et al., 2016). However, sildenafil increases H₂S release suggesting that cGMP is involved in a positive feedback mechanism regulating H₂S release. Sildenafil also causes bladder relaxation and both responses (increased H₂S and bladder relaxation) are reduced by inhibitors of CBS and CSE (Fusco et al., 2012). Further evidence for an action on smooth muscle has come from isolated tissue experiments, again on human tissues, where inhibition of H₂S synthesis does not impact detrusor contractions to carbachol directly, but does enhance bladder contractions when the urothelium is present (d'Emmanuele di Villa Bianca et al., 2016). Thus, H₂S appears to be released from the urothelium and exert at least two effects, enhancement of H₂S release and inhibition of detrusor contraction.

Prostaglandins

Prostanoid production in the bladder wall is well established, with prostanoids known to be synthesized locally within both the mucosa and smooth muscle and they are thought to play a role in the sensory arm of the micturition reflex. Urothelial production of prostanoids has been demonstrated in the bladder of most species including rat (Pinna et al., 1992, Masunaga et al., 2006, Tanaka et al., 2011), rabbit (Masick et al., 2001, Azadzoï et al., 2004), guinea pig (Saban et al., 1994, Nile et al., 2010, Guan et al., 2014) and human (Jeremy et al., 1987). Cyclooxygenase 1 is markedly localized to the urothelium and expressed within the basal and intermediate cells (de Jongh et al., 2009), although not in the umbrella cells (Rahnama'i et al., 2012). Interestingly, production of prostaglandins by the urothelium is far greater than in the suburothelium and smooth muscle (Masick et al., 2001, Azadzoï et al., 2004). The prostaglandins released are those known to be both excitatory and inhibitory in nature within the lower urinary tract and include PGE₂, PGF_{2α} as well as PGI₂ and PGD₂.

Release of prostaglandins from the bladder urothelium is induced by stretch and bladder distension (Downie and Karmazyn, 1984, Jeremy et al., 1987, Tanaka et al., 2011, Farr et al., 2017), although this may vary with pathology since PGE₂ release is not stretch-related in urothelial cancer RT4 cells (Kang et al., 2013). ATP, Ach and NO also regulate PGE₂ release in a complex signaling interaction which sees ATP (Nile et al., 2010) and Ach (via M₂ receptors) (Nile and Gillespie, 2012) stimulate prostaglandin release, whilst NO has a negative feedback effect on cholinergic release of PGE₂ (Nile et al., 2010).

Evidence for a direct influence of urothelially-released prostaglandins on the underlying smooth muscle was provided in a study by Nakahara et al., who showed that activation of protease-activated receptor-2 can stimulate release of prostaglandins from the rat bladder mucosa to contract the detrusor smooth muscle (Nakahara et al., 2003).

In summary, a number of prostaglandins are released by the urothelium, with some excitatory and some inhibitory to smooth muscle. Considering the complex pathways, multiple prostaglandins and many receptors by which these mediate responses, it is surprising how few studies appear in the literature.

Peptides & cytokines

The urothelium is also a source of inflammatory peptides and cytokines, with mediators of inflammation such as substance P stimulating the release of NGF and macrophage migration inhibitory factor (Meyer-Siegler and Vera). The release of interleukins (IL-1 β , IL-6, IL-8) has also been observed in several human urothelial cell lines including RT4, T24 and UROtsa cells following treatment with cytotoxic drugs which induce an inflammatory response (Kang et al., 2015, Farr et al., 2017). Also, in the rat, inflammation of the bladder induced by treatment with

cyclophosphamide results in the release of the cytokines IL-6 and leukemia inhibitory factor and ciliary neurotrophic factor (Girard et al., 2011). These factors released from the urothelial cells will have localized effects, but their potential effects on smooth muscle are unknown. It is unlikely NGF will have direct effects on smooth muscle contraction, since over-expression of this peptide in mice does not appear to influence the efferent arm of the micturition reflex (Girard et al., 2012). However, NGF may influence detrusor responses indirectly by altering receptor expression in smooth muscle, since urothelium-specific over-expression of NGF in mice reduces smooth muscle VPAC1 receptor transcripts, the receptor for vasoactive intestinal peptide (Girard et al., 2010). Another indirect effect of NGF on contraction may be to alter muscle mass since NGF increases expression of IGF-1 in isolated detrusor smooth muscle cells. Furthermore, after *in vivo* cyclophosphamide treatment of mice which causes muscle hypertrophy, the administration of NGF-neutralising antibody reverses the hypertrophy induced by CPO (Zhang and Qiao, 2012). The effects of these inflammatory mediators obviously require further investigation.

In summary, the known mediators released from the urothelium include ATP, Ach, prostaglandins, several peptides and the gaseous transmitters NO and H₂S. Various interactions modify release of these chemicals which complicates studies. A variety of actions have been demonstrated for most of these known mediators, but for each the evidence to support actions on detrusor smooth muscle is limited.

Urothelium derived inhibitor factor (UDIF)

Despite the number of mediators and neurotransmitters now known to be released from the urothelium, evidence as to whether any of these may act to directly influence the underlying smooth muscle of the lower urinary tract is limited, as mentioned above for each mediator. In particular, attempts to elucidate the nature of the UDIF modulating smooth muscle contractility in the bladder and urethra have been unsuccessful. In the pig bladder, the inhibitory effect of the urothelium is not due to the release of NO, nor is the UDIF a cyclo-oxygenase product, a catecholamine, adenosine, GABA or an endothelium-released hyperpolarizing factor sensitive to apamin. It is also not affected by antagonists at P₁ or P₂ purinergic receptors (Hawthorn et al., 2000). Involvement of NO, cyclo-oxygenase products, and β -adrenoceptors were similarly ruled out as candidates for the UDIF in the human bladder (Chaiyaprasithi et al., 2003), and in the guinea bladder the inhibitory factor is not NO, is not mediated by adenosine receptors nor is it a cyclo-oxygenase product (Guan et al., 2014). Similarly in the urethra the urothelial inhibition is independent of NO (Pinna et al., 1992) and cyclo-oxygenase products (Folasire et al., 2017).

In the rat, exogenous ATP suppresses detrusor contractions in a manner similar to that seen in the presence of the mucosa (Santoso et al., 2010), but there is no direct evidence that endogenous ATP inhibits contraction and purinergic antagonists such as suramin do not prevent the inhibitory effect of the mucosa in the pig bladder (Hawthorn et al., 2000). In contrast, in the rat ureter the inhibitory action of the urothelium, which prevents spontaneous contractile activity and decreases excitatory effects of carbachol, bradykinin and angiotensin II on ureteral motility, appears to involve cyclo-oxygenase products, which may activate the release of a relaxing factor (Mastrangelo and Iselin, 2007). Additionally, in the hamster urethra, NO and ATP-dependent inhibitory factors have been proposed (Pinna et al., 1996).

In terms of the putative excitatory factors released from the urothelium, which act to inhibit relaxations of the smooth muscle, these seem to be prostanoid in nature (Nakahara et al., 2003). However, in the human bladder angiotensin and neurokinin pathways may also be involved (Propping et al., 2015b), and in the pig bladder neck BK channels may play a role (Ribeiro et al., 2014). Thus, the nature of the urothelium-derived agents that have been shown to modulate smooth muscle in the lower urinary tract appear to be complex and are still far from being understood.

Pathophysiology of the urothelium

Based on the role of the urothelium in sensing the cellular environment and releasing mediators to modify it, several investigators have explored alterations of a wide range of parameters related to the morphology and function of the urothelium (Table 2). Such investigations were reported for various species including mouse (Daly et al., 2014) (Pak et al., 2010), rat (Johansson et al., 2002a, Afiatpour et al., 2003, Liu and Daneshgari, 2006, Doyle et al., 2018) (Eika et al., 1993, Haefliger et al., 2002, Pitre et al., 2002, Murray et al., 2004, Chopra et al., 2005, Tong et al., 2006, Barendrecht et al., 2007, Cheng et al., 2007, Tong and Cheng, 2007, Andersson et al., 2008, Hanna-Mitchell et al., 2013, Coelho et al., 2015, Xiao et al., 2015) (Andersson et al., 2008), rabbit (Santarosa et al., 1994, Azadzoï et al., 1999, Azadzoï et al., 2010) and human (Lowe et al., 1997, Vaidyanathan et al., 1998, Mansfield et al., 2005, Datta et al., 2010, Kumar et al., 2010, Munoz et al., 2011, Bschiepfer et al., 2012, Kurizaki et al., 2013, Ballouhey et al., 2015), as well as in vitro with cell lines derived from human urothelium (Kang et al., 2015). They involved a range of conditions including ageing (Afiatpour et al., 2003, Mansfield et al., 2005, Daly et al., 2014), overactive bladder syndrome (OAB)/idiopathic detrusor overactivity (Datta et al., 2010, Kumar et al., 2010, Munoz et al., 2011), neurogenic

voiding dysfunction (Vaidyanathan et al., 1998, Datta et al., 2010, Ballouhey et al., 2015, Doyle et al., 2018), chronic pelvic ischemia (Azadzoï et al., 1999, Azadzoï et al., 2010), bladder outlet obstruction (Santarosa et al., 1994, Haefliger et al., 2002, Johansson et al., 2002a, Barendrecht et al., 2007, Bschiepfer et al., 2012, Kurizaki et al., 2013), diabetes (Eika et al., 1993, Pitre et al., 2002, Liu and Daneshgari, 2006, Tong et al., 2006, Cheng et al., 2007, Tong and Cheng, 2007, Pak et al., 2010, Hanna-Mitchell et al., 2013, Xiao et al., 2015), and cystitis (Lowe et al., 1997, Sun et al., 2009), the latter mostly in the context of cyclophosphamide treatment (Murray et al., 2004, Chopra et al., 2005, Andersson et al., 2008, Coelho et al., 2015) (Andersson et al., 2008). Of note, it is not necessarily the abundance of a given factor but rather its ratio with that of other factors that may be of importance (Yoshida et al., 2004, Munoz et al., 2011, Silva et al., 2015). However, very few combinations of species, conditions and outcome parameters have been studied more than once, which makes it difficult to determine the robustness of any of the reported alterations.

These reports clearly demonstrate that the morphology and function of the urothelium is dynamically regulated in disease. Such alterations could plausibly contribute to the pathophysiology of bladder dysfunction and deserve additional investigation. While it is frequently speculated that such alterations could relate to the pathophysiology of the disease under investigation, it remains uncertain in most cases whether they are a consequence or a cause of alterations of the urothelium in disease. An example for this is the finding of a correlation between urothelial expression of α_1 -adrenoceptors and symptom severity in men with benign prostatic hyperplasia (Kurizaki et al., 2011).

<<<place Table 2 approximately here>>>

Therapeutic aspects

A limited number of studies have explored how common treatments of OAB may affect the urothelium. Muscarinic receptor antagonists inhibit ATP release from human bladder strips, which is largely absent after mucosal removal, implying an effect on the mucosa (Yoshida et al., 2009). Studies with isolated rat bladder also reported that muscarinic antagonists reduce ATP release and extended these findings to release of prostaglandin E₂ (Yokoyama et al., 2011). Experiments in the human urothelium-derived UROtsa cell line found increased proliferation in response to a muscarinic agonist, which was blocked by a muscarinic antagonist (Arrighi et al., 2011); such findings could provide a mechanistic explanation to increased urothelial thickness in some pathological states (see Table 2).

Botulinum toxin was reported to inhibit ATP release from cultured rat urothelial cells (Hanna-Mitchell et al., 2015) and also from mouse urothelium in vivo (Collins et al., 2013). In contrast, it promoted NO release in the latter model. It attenuated the decrease of urothelial immunoreactivity of M₁ muscarinic receptors in patients with detrusor overactivity (Datta et al., 2010). Botulinum toxin also attenuated the increase in urothelial expression of TRPV1 and NGF in rats with bladder outlet obstruction (Ha et al., 2011). In contrast, urothelial NGF overexpression in mice leads to neuronal hyperinnervation and changes in bladder function (Schnegelsberg et al., 2010). Finally, the purinergic P2Y₆ receptor agonist UDP inhibited Ach release from rat bladder strips in the presence but not absence of urothelium (Carneiro et al., 2014)

Conclusions

The urothelium plays a central role in the sensing of the filling state of the urinary bladder via the presence of neurotransmitters, paracrine mediators, hormones and other constituents of the extracellular environment (Figure 2). In response to such inputs, it can release various mediators that act in an autocrine and paracrine manner to modulate the function of the urothelium itself, but also that of other cells types including smooth muscle and afferent nerves. While the presence of the urothelium has proven a strong modulator of detrusor contractility, such an influence is apparently not mediated by known factors released from the urothelium. The molecular identity of such factors has remained elusive. It may be speculated that it is related, if not identical to, endothelium-derived relaxing factors (other than NO) and endothelium-derived hyperpolarizing factor. The morphology, sensing mechanisms and mediator release from the urothelium apparently undergo major changes with ageing, in pathological conditions and upon treatment, with the influence of gender also unknown. However, the evidence is too scattered to allow robust conclusions on the nature of these alterations and whether they represent causative parts of the pathophysiology or adaptations to an underlying pathology. Robust identification of such altereations may lead to urothelium-directed therapeutics, that allow a more targeted treatment of bladder dysfunction.

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Tables

Table 1: Model systems to study urothelial function. For details see section on methodological considerations in main text.

Model	Key advantage	Key limitation
In vivo	Physiologically most relevant	Little direct information on involvement of urothelium in bladder function
Isolated bladder strips with and without urothelium/mucosa removal	Can directly assess functional impact of urothelium	Conclusions only indirect by comparing absence and presence; unclear whether removal affects ability to sense and/or to respond to stimulus
Freshly isolated urothelial cells	Allows detailed mechanistic investigation	Effects on other cell types can only be inferred
Primary culture and cell lines	Allows mechanistic investigation with larger quantities of cells	Expression pattern markedly altered by passaging and transformation

Table 2: Parameters of urothelial morphology and function reported to be altered under various conditions. BOO, bladder outlet obstruction; NVD, neurogenic voiding dysfunction; OAB, overactive bladder syndrome

Parameter	Species	Condition	Effect	Reference
Morphology				
General morphology	rabbit	Chronic pelvic ischemia	disturbed	(Azadzoï et al., 1999, Azadzoï et al., 2010)
Apoptosis	rabbit	BOO	increase	(Santarosa et al., 1994)
Thickness	rat	BOO	increase	(Barendrecht et al., 2007)
Cross-sectional area	rat	diabetes	increase	(Xiao et al., 2015)
Umbrella cells	rat	diabetes	desquamation	(Hanna-Mitchell et al., 2013)
Bladder function				
Contraction	rat	spinal cord injury	decrease	(Doyle et al., 2018)
Receptor and channel expression				
Muscarinic M ₁	human	OAB/NVD	decrease	(Datta et al., 2010)
Muscarinic M ₂	human	aging	decrease	(Mansfield et al., 2005)

	rat	diabetes	increase	(Tong et al., 2006, Tong and Cheng, 2007)
	rat	spinal cord injury	decrease	(Doyle et al., 2018)
Muscarinic M ₃	rat	diabetes	increase	(Cheng et al., 2007, Tong and Cheng, 2007)
Bradykinin B ₁	rat	cystitis	increase	(Chopra et al., 2005)
Endothelin ET _B	rat	aging	decrease	(Afiatpour et al., 2003)
Purinergic P2X ₃	mouse	aging	increase	(Daly et al., 2014)
TRPV1	rat	diabetes	increase	(Hanna-Mitchell et al., 2013)
trkA, trkB	rat	cystitis	decrease	(Murray et al., 2004)
Mediators				
ATP release	human	OAB/NVD	increase	(Kumar et al., 2010, Munoz et al., 2011)
	human	OAB	increase	(Birder et al., 2003, Kumar et al., 2007)
	human	cystitis	increase	(Sun et al., 2009)

	human	Mitomycin C	decrease	(Kang et al., 2015)
Acetylcholine	human	Mitomycin C	increase	(Kang et al., 2015)
Prostaglandin E ₂	human	Mitomycin C	increase	(Kang et al., 2015)
NO	rat	cystitis	increase	(Andersson et al., 2008)
Other parameters				
Sphingosine kinase	human	NVD	increase	(Ballouhey et al., 2015)
Inducible NO synthase	rat	BOO	increase	(Johansson et al., 2002a)
Endothelial NO synthase	rat	BOO	decrease	(Johansson et al., 2002a)
Connexins	rat	BOO	increase	(Haefliger et al., 2002)
bFGF, TGF	rabbit	BOO	increase	(Santarosa et al., 1994)
NGF	rat	cystitis	increase	(Coelho et al., 2015)
	human	cystitis	increase	(Lowe et al., 1997)
DNA synthesis	rat	diabetes	increase	(Eika et al., 1993)

Legends to the figures

Figure 1: Effects of mucosa denudation on contractile responses of the pig urethra to agonists. Cumulative concentration-response curves to carbachol (upper panel, $n = 5$), noradrenaline (middle panel, $n = 4$) and phenylephrine (lower panel, $n = 7$) on isolated porcine urethral strips prepared either with an intact mucosa (filled circles) or with the mucosa removed (open squares). Data are shown as means \pm SD. Differences in E_{max} between intact and denuded strips were statistically significant ($P < 0.05$) in unpaired, two-tailed t-tests. Adapted with permission from (Folasire et al., 2017).

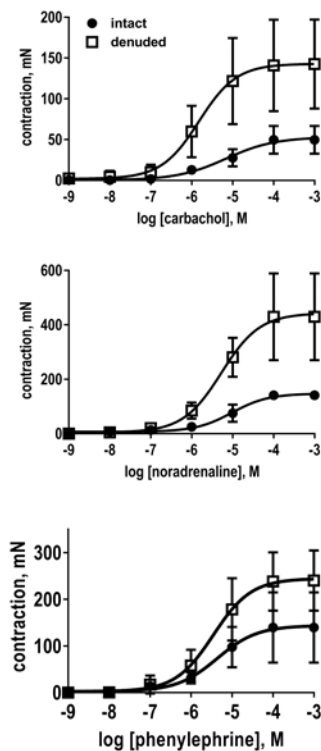


Figure 2: Neurotransmitter and hormonal inputs to the urothelium and outputs to the smooth muscle. The urothelium has receptors for a wide variety of transmitters including nerve growth factor (NGF), prostaglandins (PG), acetylcholine (ACh), substance P (SP), neurokinin A (NKA), adenosine triphosphate (ATP), 5-hydroxytryptamine (5HT), noradrenaline (NA), bradykinin (BK), capsaicin (Cap), angiotensin II (AG), cannabinoids (CB) and endothelin (ET). In response to these inputs, the urothelium releases a number of chemical mediators including ciliary neurotrophic factor (CNF), macrophage migration inhibitory factor (MIF), leukocyte inhibitory factor (LIF), hydrogen sulphide (H₂S), urothelium-derived inhibitory factor (UDIF), and interleukins (IL). These actions of these mediators on smooth muscle can be excitatory [+], inhibitory [-] or unknown [?].

