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Moro, Christian; Phelps, Charlotte

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Urothelium removal does not impact mucosal activity in response to muscarinic or adrenergic receptor stimulation.

Christian Moro* and Charlotte Phelps.

Centre for Urology Research, Faculty of Health Sciences and Medicine, Bond University, QLD 4229, Australia

* Corresponding author:

Christian Moro

Faculty of Health Sciences & Medicine, Bond University, Robina, QLD, 4229, Australia

Email: cmoro@bond.edu.au

Author ORCiD

Christian Moro: https://orcid.org/0000-0003-2190-8301

Charlotte Phelps: 0000-0002-4217-0214

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Abstract

The inner lining of the urinary bladder (urothelium and lamina propria, or bladder mucosa) has an important role as a tissue barrier between stored urine and the underlying smooth muscle, as well as in the modulation and regulation of bladder contractility. However, the individual influence of the apical urothelial layer on the contractile activity of this tissue is uncertain. The aim of this experiment was to identify the contractile activity of the lamina propria after removal of the urothelium. Several methods were used to mechanically disrupt the urothelium, including dabbing the tissue with a paper towel, longitudinal swipes with a cotton bud, or a longitudinal scrape with the edge of a scalpel. Hematoxylin-eosin staining was utilized to determine the level of removal of the apical urothelial cells. Spontaneous contractile activity was measured in organ baths, and responses to the agonists carbachol and isoprenaline were obtained. Three longitudinal swipes with a cotton bud was found to be the optimal method to remove the majority of the urothelium without damaging the lamina propria. Upon removal of the urothelium, the spontaneous activity of the tissue was unaltered. Similarly, responses to carbachol (1 μM) and isoprenaline (1 μM) were not affected after removal of the urothelium. The urothelium can be effectively removed without damaging the lamina propria. This apical tissue layer is not responsible for mediating the increases to spontaneous phasic activity or tonic contractions of the bladder mucosa (urothelium with lamina propria) when muscarinic or adrenergic receptors are stimulated. This research presents the lamina propria as the important cell layer mediating the overall contractile activity of the bladder wall.

Keywords: bladder contraction; bladder histology; bladder mucosa; lamina propria; submucosa; urinary bladder; urothelium.

Introduction

The urothelium is one of the most effective permeability barriers to protect the body from the toxic substances of the urine, with a high transepithelial electrical resistance and ability to accommodate significant changes in the surface area during bladder filling (Wu et al., 2009). It is composed of three morphologically distinct cell layers, including basal cells, intermediate cells, and umbrella cells which cover the superficial apical surface. A range of mechanisms work together to effectively maintain the urothelial barrier. The glycosaminoglycan (GAG) layer lines the luminal surface of the urothelium and the basal membrane separates the urothelium from the underlying connective tissue layer. Approximately 90% of the apical surface of umbrella cells are covered by proteins called uroplakins, which function to maintain the integrity and strength of the urothelium, prevent ruptures during the filling phase, protect against toxic substances, and contribute to the low permeability of water and solutes (Negrete et al., 1996). The GAG layer contributes to the maintenance of the barrier function from the harmful substances found in the urine, and damage to it can lead to exposure of urine to the underlying epithelial cells (Hurst et al., 1987). In addition, each cell in the urothelium maintains tight junctions between adjacent cells (Peter, 1978), which forms a physical barrier that is impermeable to solvents in the urine.

The urothelium also releases several chemical mediators, such as ATP (Durnin et al., 2019; Yu, 2015), acetylcholine (Moro et al., 2011), and nitric oxide (Moro et al., 2012), which play a role in sensory signalling of the bladder (Merrill et al., 2016). This wide range of properties identifies the urothelium as an important cell layer in the overall regulation of bladder activity. We have previously shown the contractile activity of the urothelium/lamina propria (Moro et al., 2012; Phelps et al., 2022), yet there is no clear evidence for a contractile-

mediating cell type in the urothelium, suggesting the activity likely arises from the lamina propria.

Past studies have attempted to remove the inner lining from *in vitro* preparations of bladder while leaving the detrusor intact. Examples of this include both pig (Hawthorn et al., 2000) and human samples (Chaiyaprasithi et al., 2003; Kumar et al., 2004). However, the methods employed resulted in the removal of the urothelium and lamina propria together, rather than just the urothelium. In order to assess the influence of removal of the urothelium on contractility of the urothelium/lamina propria, a method is required that removes only the apical layers.

One method for removing only the endothelial cells from tissue preparations is by dabbing the surface with a paper towel, such as that used in blood vessels (Sade & Folkman, 1972). An alternative method involves gently rubbing the surface, as commonly used to remove the endothelium from blood vessels (Buchwalow et al., 2008). This latter study utilised a cotton-covered wire, although a variety of other tools have been used such as a wooden dowel (Moore et al., 2005), or other forms of mechanical debridement (Baxter, 1995; Furchgott & Zawadzki, 1980; Venugopalan et al., 1997). One study utilised an adapted method in rat bladders, gently swiping the internal lining of the bladder with a cotton bud to remove the urothelium (Munoz et al., 2010). Demonstration of this method was effective in removing the urothelium without specifically damaging the underlying lamina propria. Alternatively, another study on pig bladder tissue removed only the urothelium by scraping the epithelial surface with a scalpel (Cheng et al., 2011).

The present study aimed to identify which of the previously published methods is optimal for disrupting the majority of the porcine apical urothelial cells without damaging the underlying lamina propria. In addition, removing the urothelium may influence the contractile activity identified in intact urothelial/lamina propria strips. This was investigated by measurements of spontaneous activity and the responses to agonists, to distinguish if the pacemaker activity in the isolated tissue samples is inhibited following disruption to the urothelium.

Materials and methods

Tissue collection

Fresh bladders from Large White-Landrace-Duroc female pigs (6 months old, ~80 kg) were obtained from a local abattoir and immediately immersed in cold Krebs-bicarbonate solution (composition in mM: NaCl 118.4; $NaHCO_3$ 24.9; $CaCl_2$ 1.9; $MgSO_4$ 1.15; KCl 4.7; KH_2PO_4 1.15; and D-glucose 11.7). Bladders were obtained from the local abattoir after slaughter for the routine commercial provision of food, and as no animals were bred, harmed, culled, interfered, or interacted with as part of this research project, animal ethics approval was not required. All tissues were used within three hours of the animal's slaughter.

Mechanical disruption of the urothelium

Strips of urothelium/lamina propria were carefully dissected with fine scissors from the wall of the bladder dome. Tissue strips were continually washed with cold Krebs-bicarbonate solution during the preparation and dissection stage. Once isolated urothelium/lamina propria strips of tissue were prepared, several different methods were used to disrupt the urothelium. This included: dabbing the tissue with a paper towel; a single longitudinal swipe with a cotton bud; two longitudinal swipes with a cotton bud; three longitudinal swipes with a cotton

bud; four longitudinal swipes with a cotton bud; or a longitudinal scrape with the edge of a scalpel.

Histological preparations

Immediately after preparation of the bladder samples, the separate tissues were fixed in 10% Neutral Buffered Formalin (Fronine, QLD, AUS) and left for 24 hours. Following this, bladders were prepared for sectioning and staining by embedding in paraffin wax. Histology was performed using hematoxylin-eosin staining to determine the level of removal of the apical urothelial cells.

Functional organ bath studies

For each sample, strips of urothelium with lamina propria were prepared (20 x 5 mm) and immersed in Krebs-bicarbonate solution, maintained at 37°C and gassed with 5% CO_2 in oxygen. The tissues were attached to isometric force transducers (ADInstruments MCT050/D) and tensions recorded with a Powerlab system using Labchart v7 software (ADInstruments, Castle Hill, Australia). After washing with fresh Krebs solution, the tissues were allowed to equilibrate for 45 minutes under a baseline tension of ~2g.

In separate experiments, samples of urothelium/lamina propria were dissected into 4 cm strips and cut in half. The two 2 cm strips were set up in pairs, with one remaining as control (intact), and one swiped three times with a cotton bud to disrupt the urothelium. Frequency (cycles min^{-1}), and tension (g) responses were obtained in the absence and presence of carbachol (1 µM, $n = 8$) and isoprenaline (1 µM, $n = 8$). Responses to agonists were taken at the peak response and data obtained was analysed using paired Student's *t*-tests (where $p < 0.05$ was considered statistically significant).

Results

Histological studies

In whole segments of the bladder dome stained with hematoxylin-eosin, the urothelium, lamina propria and underlying detrusor were evident. Following removal of the detrusor muscle, the urothelium and lamina propria were not damaged from the dissection process. The umbrella cells were clearly intact and the process of preserving the tissues by fixation and embedding in paraffin wax was a successful method to retain the individual layers (Figure 1).

Several methods were employed to remove the urothelial layer without varying results. An attempt was made to remove the urothelium using a method previously used on vascular endothelium, outlined by Sade and Folkman (1972). Tissues were dabbed with a "Kimwipe" paper towel two times, however after this process, there was no observable difference between the urothelial cells of these tissues and those of controls.

Similarly, the effect of a single or double longitudinal swipe with a cotton bud, a method adapted method from Munoz et al. (2010), had little effect on tissue structure other than a very slight compressing of the urothelium with this method, although the apical cell layers were still intact and not removed from the tissue. Two longitudinal swipes with a cotton bud was effective at removing some portions of the urothelium, although the success was inconsistent. There were also regions of the apical layer which were left relatively intact (Figure 1). Three longitudinal swipes with a cotton bud removed a large proportion of the urothelium down to the basal layers, producing a substantial disruption to the urothelium but leaving the lamina propria largely intact. Four swipes with cotton buds across the urothelial

surface resulted in a clear and uniform disruption of the urothelium which was generally consistent throughout the samples. However, portions of the lamina propria were also disrupted, and large sections of the tissue underwent damage that affected the lamina propria.

The effect of longitudinal scraping of the tissue with the edge of a scalpel caused damage to both the urothelium and the lamina propria. Although the majority of damage was observed along the urothelium, in some sections the scalpel broke through the urothelium and damaged the lamina propria. In these sections the scalpel had dug directly into the lamina propria, causing severe damage to large numbers of cells within.

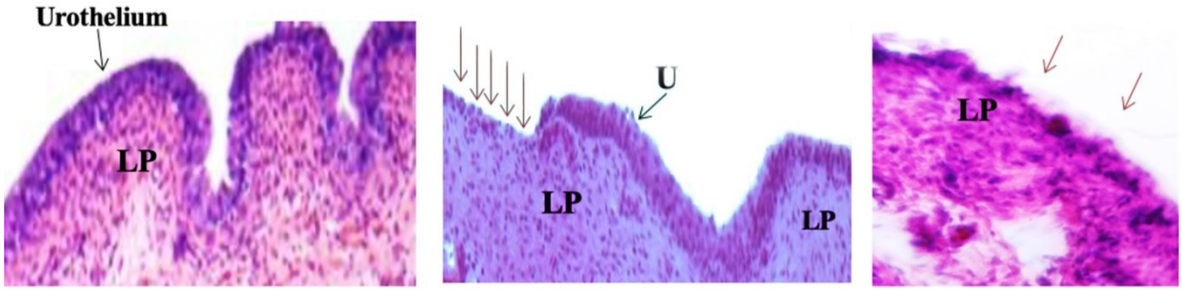

Figure 1: Haematoxylin-eosin staining of urothelium and lamina propria urinary bladder strips. **Left image**: Control urothelium/lamina propria tissues after dissection from the detrusor muscle. The urothelium (arrows) and lamina propria (LP) were easily identifiable and not damaged by the dissecting procedure. **Middle image**: Effect of two longitudinal swipes with a cotton bud, showing disruption to parts of the urothelium (arrows) but with large sections of the layer remaining intact. **Right image:** The undamaged lamina propria after complete urothelium removal from three swipes with a cotton bud (magnification: 100x).

Functional isolated tissue bath responses after three swipes with a cotton bud

Upon histological analysis, three swipes with a cotton bud was found to be the optimal method to successfully disrupt the urothelium without damaging the underlying lamina propria. This method was used for subsequent functional studies. There was no significant difference in the frequency of spontaneous phasic contractions or tonic contractions between the control tissues and those that underwent urothelium removal with three swipes of a cotton bud (3.06 ± 0.22 cycles min^{-1} vs 2.99 ± 0.26 cycles min^{-1} for frequency, 1.57 ± 0.18 g vs 1.57 ± 0.16 g for tonic contractions, n = 8).

Response to carbachol

After a 60-minute equilibration period, responses were obtained to carbachol (1 µM, n = 8). The increase in spontaneous phasic contractile frequency induced by carbachol was similar between the control and urothelium-denuded tissues (2.02 ± 0.80 cycles min^{-1} vs 1.55 ± 0.32 cycles min^{-1}, Figure 2). In addition, the increase in basal tension to carbachol was also similar in the two groups of tissues (3.27 ± 0.37 g vs 3.31 ± 0.45 g, Figure 3).

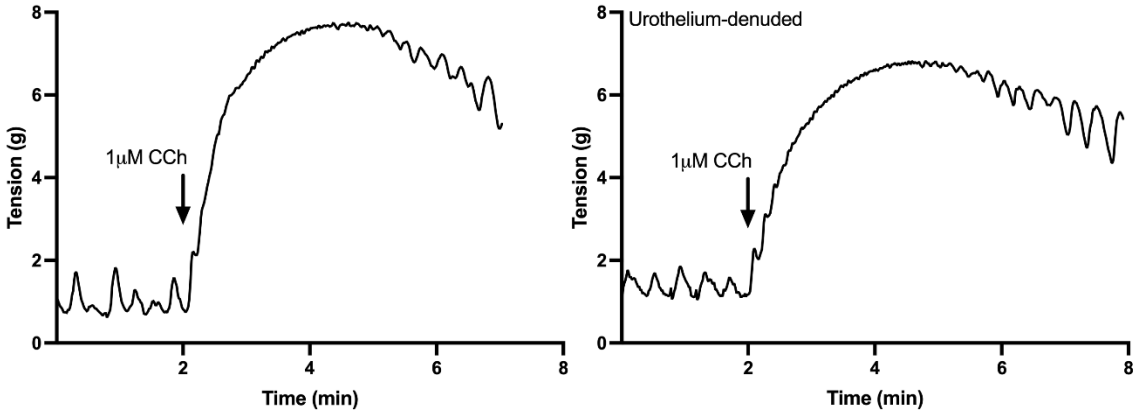

Figure 2. Left: Representative trace depicting the baseline spontaneous contractile activity and the response to carbachol (1 µM) in the control tissue. **Right:** Representative trace depicting the baseline spontaneous contractile activity and the response to carbachol (1 µM) in a tissue that had undergone longitudinal swiping three times with a cotton bud.

Response to isoprenaline

Isoprenaline (1 µM, n = 8) had a clear effect on the bladder, causing strong relaxations of the tissue. The decrease in contractile frequency induced by isoprenaline was similar in control and urothelium-denuded tissues (0.88 ± 0.18 cycles min^{-1} vs 1.15 ± 0.22 cycles min^{-1}). In addition, the decrease in basal tension to isoprenaline was not significantly different between the two groups of tissues (1.01 ± 0.07 g vs 1.35 ± 0.16 g, Figure 3).

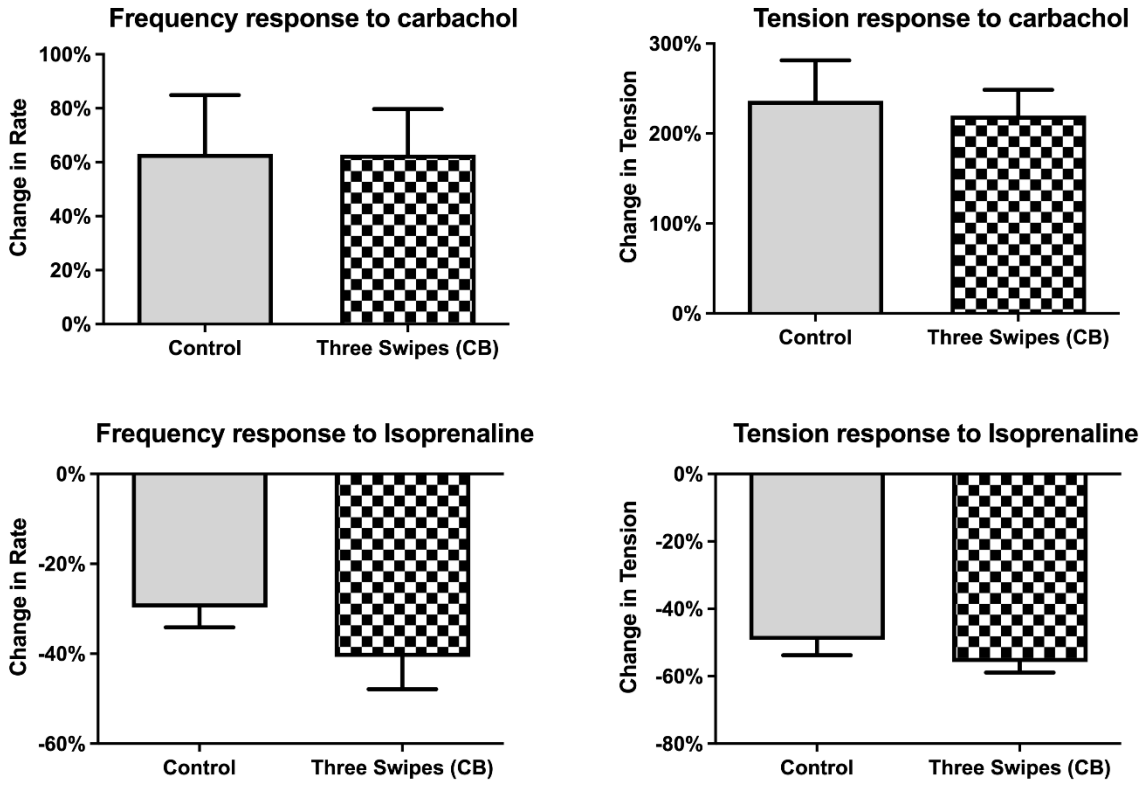

Figure 3: Upper figures: The effect of carbachol (1 µM, n = 8) on tissues that had undergone three swipes with a cotton bud (CB) compared to controls. There was no significant difference in the spontaneous contractile frequency or the tension response to carbachol. **Lower figures**: The effects of isoprenaline (1 µM, n = 8) on the tissues after three swipes with a cotton bud. There was no significant difference in the spontaneous contractile frequency or the relaxation responses to isoprenaline.

Discussion

Isolated strips of the urothelium and lamina propria develop spontaneous phasic contractions which can be modulated via activation of the muscarinic (Moro et al., 2011), adrenergic (Moro et al., 2013), histaminergic (Stromberga et al., 2019), serotonergic (Moro et al., 2016), and prostaglandin (Stromberga et al., 2020) receptor systems. However, the mechanisms underlying this activity remain unclear and it is not known how the presence of urothelium influences the overall activity of the urinary bladder. It is likely that the cells involved in the generation, coordination, and contractile function of the spontaneous activity lie within the lamina propria rather than in the urothelium. Although still not fully supported, this hypothesis has also been suggested by authors of prior studies examining this layer (Cheng et al., 2011; Fry et al., 2012; Ikeda et al., 2007).

It is known that removal of the urothelium/lamina propria affects detrusor contractions. This has been shown in a study where contractile responses to electrical field stimulation in the feline bladder were significantly increased following urothelium/lamina propria removal (Levin et al., 1995). Additionally, isolated pig bladder strips also demonstrated increased contractile responses to muscarinic receptor stimulation after urothelium/lamina propria removal, and this is thought to be due to an unknown urothelium derived inhibitory factor (Hawthorn et al., 2000). Removal of the urothelium from rat bladder strips does not appear to affect basal release of nitric oxide or ATP (Munoz et al., 2010). However, in that study, urothelial removal did produce a significant reduction in carbachol-induced release of nitric oxide and electrical-field stimulated release of ATP. It was unclear whether these results related functionally to the spontaneous contractile activity of urothelium/lamina propria or solely on the contractile ability of the detrusor smooth muscle.

In an attempt to disrupt the urothelium while leaving the lamina propria intact, previously published methods for removal of this cell layer were investigated. Histological examination showed that one or two dabs with a paper towel had no effect on the urothelium. Although past studies have used this method successfully in vascular endothelium (Sade & Folkman, 1972), it does not appear to influence the bladder urothelium to the same effect. This is most likely due to the presence of a squamous epithelium in blood vessels compared to a transitional epithelium on the urothelium.

Scraping with a scalpel, which has been used previously on porcine bladders (Cheng et al., 2011), did effectively disrupt the urothelium. However, this method appeared to 'tear' away sections of the lamina propria. It is possible that careful use of a scalpel blade, giving consideration to the angle and direction of the blade, would be an effective method for collection of urothelial cells or removing the urothelium from small areas of the tissue.

Mechanical methods for removing the apical layers via swabbing or rubbing the epithelial surface have also been used successfully in a range of tissues (Buchwalow et al., 2008; Furchgott & Zawadzki, 1980). Additionally, longitudinal swiping has been successful in rat urinary bladder (Munoz et al., 2010). One or two swipes with a cotton bud were ineffective at removing large proportions of urothelium from the luminal surface in the present study. This may differ from that experienced in vascular studies, due to the larger and potentially more robust nature of the porcine bladder urothelium in comparison to the endothelium in rat bladders or blood vessels. However, three longitudinal swipes with a cotton bud consistently disrupted the urothelial layer across the tissue samples. This method was less invasive and damaging to the lamina propria than scraping with a scalpel. After this success, four swipes

with a cotton bud were attempted, but this method began to impact the underlying lamina propria.

Upon confirming the validity of the removal method, paired functional experiments were performed to identify the impact of three swipes with a cotton bud on the lamina propria contractile activity. The basal spontaneous frequency was evident in both control and urothelium-removed samples and not altered by urothelium removal. In addition, responses to muscarinic receptor stimulation or β-adrenoceptor stimulation were also not affected by urothelial removal. This suggests that the urothelium is not involved in mediating the spontaneous contractile activity of the urothelium/lamina propria. This was interesting, as urothelial cells are known to express a range of receptors such as vanilloid and adrenergic receptors (Birder et al., 2001), as well as G-protein coupled receptors, such as muscarinic receptors (Wang et al., 1995). Additionally, the urothelium can release a number of chemical mediators including acetylcholine (Yoshida et al., 2006), nitric oxide (Birder et al., 1998), and ATP (Ferguson et al., 1997). Although some of these, such as ATP, can also be released from the lamina propria (Sadananda et al., 2012), it was interesting to identify a lack of effect after removal of the urothelium.

A diverse collection of cells also resides within the lamina propria, which may induce the contractile activity of this layer. These include connective tissue, blood vessels, and afferent nerve fibres, as well as a range of cells types which include interstitial cells and fibroblasts (Woodman et al., 2011). Additionally, a variety of immune cells are present in the lamina propria, such as mast cells and dendritic cells (Christmas & Rode, 1991; Kummer et al., 2007). Within this layer, the interstitial cells most likely mediate contractile activity and the responses to receptor activation. It is understood that these cells have the ability to contract

and hold close associations with the calcium transients and spontaneous activity (Fry et al., 2012). Interstitial cells have also previously been suggested as the cell type mediating urothelial/lamina propria contractions in response to neurokinin-A (Sadananda et al., 2008). Therefore, it appears from past research that the interstitial cells are likely modulators for the observed responses demonstrated in strips of urothelium-removed lamina propria.

Limitations

Although most of the urothelium was removed from the cotton bud swipes, only certain sections of the tissue were assessed through histology. In some cases, small amounts of urothelium remained. This means that there may have been some influence from the urothelium in the contractile responses observed. There is also evidence to support the urothelium undergoing some cell detachment during desquamation, with a potential role of junctional complexes (Veranic & Jezernik, 2000). As such, some consideration could be made regarding what happens after the tissue layer has been mechanically disrupted, rather than immediately following the damage. In addition, this study does not address the modulatory role of the urothelium under physiological conditions. Future experiments could assess the influence of this tissue layer at different baseline tones, as well as under stretch or tactile stimuli. Due to the addition of agonists to the bath, this study did not assess the influence of signalling molecules released from the urothelium itself. These molecules might hold a clear role in the contractile nature in response to physiological stimuli.

Conclusions

In conclusion, disruption to the apical urothelial cells of the pig bladder could be accomplished by a swipe with a scalpel or swiping three times with a cotton bud, but not by dabbing with a paper towel. The optimal method for urothelium removal was determined to

be three gentle swipes with a cotton bud. Disruption to this layer did not affect the rate of spontaneous contractions, nor the response to muscarinic receptor stimulation and the response to β-adrenoceptor activation of the underlying tissue. This indicates that the phasic activity of the urothelium/lamina propria most likely arises from cells within the lamina propria and is independent of urothelial regulation.

Declaration of interest

The authors report there are no competing interests to declare.

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Figure captions

Figure 1: Haematoxylin-eosin staining of urothelium and lamina propria urinary bladder strips. **Left image**: Control urothelium/lamina propria tissues after dissection from the detrusor muscle. The urothelium (arrows) and lamina propria (LP) were easily identifiable and not damaged by the dissecting procedure. **Middle image**: Effect of two longitudinal swipes with a cotton bud, showing disruption to parts of the urothelium (arrows) but with large sections of the layer remaining intact. **Right image:** The undamaged lamina propria after urothelium removal from three swipes with a cotton bud (magnification: 100x).

Figure 2. Left: Representative trace depicting the baseline spontaneous contractile activity and the response to carbachol (1 μM) in the control tissue. **Right:** Representative trace depicting the baseline spontaneous contractile activity and the response to carbachol (1 μM) in a tissue that had undergone longitudinal swiping three times with a cotton bud.

Figure 3: Upper figures: The effect of carbachol (1 μM, $n = 8$) on tissues that had undergone three swipes with a cotton bud (CB) compared to controls. There was no significant difference in the spontaneous contractile frequency or the tension response to carbachol. **Lower figures**: The effects of isoprenaline (1 μM, $n = 8$) on the tissues after three swipes with a cotton bud. There was no significant difference in the spontaneous contractile frequency or the relaxation responses to isoprenaline.