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Christian Moro  
*Bond University, Christian_Moro@bond.edu.au*

Jumpei Uchiyama  
*Kochi University*

Russ Chess-Williams  
*Bond University, Russ_Chess-Williams@bond.edu.au*

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Urothelial/lamina propria spontaneous activity and the role of M3 muscarinic receptors in mediating rate responses to stretch and carbachol

Christian Moro\textsuperscript{a} BSc

Jumpei Uchiyama\textsuperscript{b} PhD

Russ Chess-Williams\textsuperscript{a}\textsuperscript{*} PhD


\textsuperscript{a}Department of Biomedical Science, Bond University, Queensland, Australia.

\textsuperscript{b}Department of Microbiology and Infection, Kochi University, Japan.

* Corresponding author: Professor R. Chess-Williams, Department of Biomedical Science, Faculty of Health Sciences & Medicine, Bond University, Queensland 4229, Australia
Tel: +61 (0)7 55954420
Fax: +61 (0)7 55954538
Email: rchesswi@bond.edu.au

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**Statement of conflicts of Interest**

The authors state no conflict of interest.
Abstract:

Objectives: To investigate the effects of tissue stretch and muscarinic receptor stimulation on the spontaneous activity of the urothelium/lamina propria and identify the specific receptor subtype mediating these responses.

Methods: Isolated strips of porcine urothelium with lamina propria were set up for in vitro recording of contractile activity. Muscarinic receptor subtype selective antagonists were used to identify the receptors influencing the contractile rate responses to stretch and stimulation with carbachol.

Results: Isolated strips of urothelium with lamina propria (U&LP) developed spontaneous contractions (3.7 cycles min⁻¹) that were unaffected by tetrodotoxin, L-NNA or indomethacin. Carbachol (1µM) increased the spontaneous contractile rate of these tissue strips by 122 ± 27% (P<0.001). These responses were significantly depressed in the presence of the M3-selective muscarinic antagonist 4-DAMP (10-30nM), but not affected by the M1-selective antagonist pirenzepine (30-100nM) or the M2-selective antagonist methoctramine (0.1-1µM). Stretching of the tissue also caused an increase in spontaneous contractile rate and these responses were abolished by atropine (1µM) and low concentrations of 4-DAMP (10nM). Darifenacin, oxybutynin, tolterodine and solifenacin (1µM) all significantly depressed frequency responses to carbachol (1µM).

Conclusions: The urothelium with the lamina propria exhibits a spontaneous contractile activity which is increased during stretch. The mechanism appears to involve endogenous acetylcholine release acting on M3 muscarinic receptors. Anticholinergic drugs used clinically depress responses of these tissues and this mechanism may represent an additional site of action for these drugs in the treatment of bladder overactivity.
Introduction

Detrusor overactivity is a common condition affecting up to 17% of the population (Milsom et al., 2001). In this condition spontaneous bladder contractions occur during the filling stage of the micturition cycle, but the mechanisms involved are unknown. Local mechanisms appear to be involved since spontaneous contractile activity of isolated tissues has been observed (Jiang et al., 2005). In recent years the urothelium and the underlying lamina propria (here abbreviated to U&LP) have been recognized as important regulators of bladder activity, releasing factors that modulate detrusor contraction (Hawthorn et al., 2000, Templeman et al., 2002) and sensory nerve activity (Cockayne et al., 2000). Furthermore, these roles may be clinically relevant since the inhibitory effect these tissues have on detrusor contraction is depressed in the neurogenic overactive bladder (Chess-Williams, 2009), while non-neuronal ATP release from this tissue is enhanced in the bladders of patients with painful bladder (sensory) syndrome (Kumar et al., 2007). Thus the urothelium and lamina propria are known to play important roles in maintaining normal bladder function and are associated with the development of pathological states.

In addition to releasing ATP and other mediators, the U&LP of the pig bladder (Sadananda et al., 2008) and the lamina propria of the rabbit urethra (Mattisson et al., 1985, Zygmunt et al., 1993) display contractile properties and have been shown to contract in response to a number of agonists. It is presently unclear which specific cell types mediate contraction, but it has been suggested that myofibroblasts may be involved (Sadananda et al., 2008). In addition, it has been reported that spontaneous contractions of intact bladder strips may be linked to the U&LP (Akino et al., 2008). Also, these tissues from the dome of the pig bladder have been shown to develop spontaneous phasic contractile activity (Moro and Chess Williams, 2010).
Again it is unclear which cell types might act to initiate this spontaneous activity, but myofibroblasts in the lamina propria have been shown to develop spontaneous depolarisations and calcium transients, which may suggest a possible pacemaker role (Kanai et al., 2007). The present study has examined the contractile activity of isolated strips of U&LP with the aim of identifying the muscarinic receptor subtype responsible for regulating the frequency of spontaneous contractions and to investigate the effects of tissue stretch on the spontaneous rate of contraction.
Material and Methods

Fresh bladders from Large-White Landrace pigs (6 months old, 80Kg) were obtained from a local abattoir and immediately immersed in cold Krebs-bicarbonate solution (composition in mM: NaCl 188.4, NaHCO₃ 24.9, CaCl₂ 1.9, MgSO₄ 1.15, KCl 4.7, KH₂PO₄ 1.15 and D-glucose 11.7). The bladders were opened longitudinally and full thickness strips of anterior wall from the dome region were removed. From these tissues, strips of urothelium including the lamina propria were prepared (2cm x 5mm). For smooth muscle experiments detrusor strips were examined after removal of the urothelium and lamina propria. All tissues were immersed in Krebs-bicarbonate solution, maintained at 37°C and gassed with 5% CO₂ in oxygen.

The tissues were attached to isometric force transducers (ADInstruments MCT050/D) and tension recorded with a Powerlab system (ADInstruments, Castle Hill, Australia) using Labchart v7 software. After setting to a baseline tension of 2g the tissues were washed several times with fresh Krebs solution and allowed to equilibrate for 45 minutes before starting drug additions. All antagonists were left to incubate for at least 20 minutes in the organ baths prior to further agonist additions. In stretch experiments, tissues (with or without the selective antagonist) were stretched by increasing the length of the tissue by 75% and the spontaneous contractile frequency was recorded before and after stretching.

Measurements of frequency, amplitude and baseline tension were taken at the peak response after the addition of each drug or stretching. The frequency of contractions was expressed as the number of phasic waves per minute and the amplitude as grams tension. The baseline tension was taken as the lowest point of the spontaneous phasic contractions. Mean (±SEM)
values in the absence and presence of drugs were compared using Students two-tailed paired t-test with P<0.05 being taken as statistically significant. Prism software (GraphPad, San Diego, CA, USA) was used for statistical analysis of data.

Acetylcholine chloride (Ach), carbamoylcholine chloride (carbachol), α,β-methyleneATP lithium (αβmATP), tetrodotoxin (TTX), atropine sulphate, Nw-nitro-L-arginine (L-NNA), pirenzepine dihydrochloride, 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP), methoctramine hemihydrate, indomethacin and oxybutynin chloride were purchased from Sigma (St Louis, MO, USA). Darifenacin hydrobromide, solifenacin succinate and tolterodine tartrate were gifts from Astellas. Indomethacin was dissolved in 100% ethanol and control experiments were performed using vehicle alone. All other drugs were dissolved in distilled water and diluted in Krebs-bicarbonate solution.
Results

*Spontaneous phasic contractions*

The preparations of urothelium and lamina propria (U&LP) exhibited spontaneous contractions within 10 minutes of being placed in the organ bath. This regular phasic activity occurred at a spontaneous contractile frequency of $3.72 \pm 0.12$ cycles min$^{-1}$ with an amplitude of $0.71 \pm 0.05$g ($n=53$, Figure 1-1). The frequency and amplitude of spontaneous contractions, and the baseline tension of tissues, were not affected by either tetrodotoxin (1µM, $n=12$), indomethacin (5µM, $n=7$) or L-NNA (100µM, $n=10$). In contrast, only 25% of detrusor smooth muscle strips ($n=28$) set up under identical conditions, developed spontaneous activity and the spontaneous rate of the active tissues ($1.57 \pm 0.20$ cycles min$^{-1}$) was significantly lower than that of the U&LP strips ($P<0.001$).
Figure 1-1: Spontaneous activity of isolated U&LP strips; upper trace in the absence of any drug and lower trace in the presence of carbachol (1µM). Carbachol induced increases in baseline tension, but also increased the frequency of spontaneous contractions.
**Muscarinic receptor stimulation**

The frequency of spontaneous U&LP contractions was not affected by atropine (1-30µM, n=16). Addition of acetylcholine (1µM) however, increased the frequency by 39 ± 14% (n=8, P<0.05). Carbachol (1µM) produced a greater increase in frequency than acetylcholine (1µM), increasing the spontaneous rate by 122 ± 27% (Figure 1-1). Carbachol (1µM) also increased the baseline tension by 152 ± 13% (P<0.001, n=27) and the amplitude of spontaneous phasic contractions was also reduced by 47 ± 4%, (P<0.001, n=27). Increases in frequency induced by carbachol (1µM) were not affected by the addition of indomethacin (5µM) or L-NNA (100µM).

To identify which muscarinic receptor subtype mediated the increase in U&LP spontaneous rate, responses to carbachol (1µM) were obtained in the presence of receptor-selective antagonists (Figure 1-2). The M3-selective antagonist 4-DAMP (10nM) had the greatest effect on responses, more than halving the carbachol-induced (1µM) increase in frequency from 83 ± 21% to 35 ± 8% (P<0.01 n=12). A higher concentration of 4-DAMP (30nM) further depressed responses to carbachol (1µM, 18 ± 4%, n=8). The M1-receptor selective antagonist pirenzepine at concentrations up to 100nM had no significant affect on the responses to carbachol (n=12). At a concentration of 100nM (n=9) the M2-receptor selective antagonist methoctramine had no effects on the frequency or tension responses to carbachol, but at a higher concentration (1µM, n=8) it reduced the tension response without significantly affecting the U&LP frequency response (Figure 1-2).
Figure 1-2: Increases in U&LP contractile frequency induced by carbachol (1µM). Responses were obtained to carbachol in the absence (open columns) and in the presence of antagonists (shaded columns). Upper panels show changes in frequency (cycles min⁻¹) and baseline tension (grams) to carbachol in the absence and presence of 30nM and 100nM pirenzipine (M1 selective antagonist); middle panels show the effects of 100nM and 1µM methoctramine (M2 selective antagonist) and lower panels show the effects of 10nM and 30nM 4-DAMP (M3 selective antagonist). *P < 0.05, **P < 0.01, ***P < 0.001.
The effect of clinical anti-cholingergics on the muscarinic response to carbachol

At a concentration of 100nM darifenacin, oxybutynin, and tolterodine significantly reduced the frequency and tension responses of U&LP strips to carbachol (1µM). Solifenacin (100nM) slightly inhibited the response to carbachol, although this was not statistically significant at this concentration. At a concentration of 1µM all four antagonists significantly depressed the U&LP frequency and tension responses to carbachol (Figure 1-3).
Figure 1-3: Effects of clinically used anti-muscarinic drugs on frequency (left panels) and tension responses (right panels) of isolated U&LP strips to carbachol (1µM). Increases in urothelial frequency (cycles min\(^{-1}\)) and tension (grams) induced by carbachol are shown in the absence (open columns) and in the presence of antagonists (shaded columns) at concentrations of 100nM (upper panels) and 1µM (lower panels). *P < 0.05, **P < 0.01, ***P < 0.001.
In separate experiments, stretching the U&LP strips elicited an increase in the rate of spontaneous phasic contractions of $19 \pm 3\%$ (P<0.001) and baseline tension also increased from $1.75 \pm 0.12g$ to $9.58 \pm 0.80g$ (P<0.001, n=42). The increase in frequency of phasic contractions induced by stretch was not significantly altered after desensitisation of $P_{2\alpha}$ receptors with αβmATP (10µM, n=12), the presence of the NOS inhibitor L-NNA (100µM, n=7) or the cyclooxygenase inhibitor indomethacin (5µM, n=12). However the stretch-induced increase in frequency was abolished by atropine (1µM, n=11, P<0.01, Figure 1-4), indicating the involvement of muscarinic receptors. To identify which receptor mediated this response, the frequency response to stretch was examined in the presence of selective antagonists (Figure 1-4). In the presence of 4-DAMP (10nM, n=7) the increase in frequency observed during stretch was abolished. In contrast, pirenzepine (100nM, n=7) and methoctramine (100nM, n=8) had no effect on the stretch-induced increase in phasic contractions.
Figure 1-4: Upper panels: Increases in the frequency of spontaneous contractions of isolated strips of U&LP when stretched in the absence (open columns) and presence (shaded columns) of atropine (1µM), L-NNA (100µM) or indomethacin (5µM). Responses are expressed as the percentage increase in frequency induced by stretch compared to those in the absence of drug. Lower panels: Effects of muscarinic receptor subtype selective antagonists on the frequency responses of U&LP strips to stretch. The spontaneous frequency before (open columns) and after stretching (solid columns) was obtained in the absence and then presence of either pirenzepine (M1-selective), methoctramine (M2-selective) or 4-DAMP (M3-selective). Stretching significantly increased the frequency of spontaneous U&LP contractions in all groups, except in the presence of 4-DAMP *P < 0.05, **P < 0.01.
Discussion

Contraction of the lamina propria was first reported for tissues from the urethra of the rabbit (Mattiasson et al., 1985, Zygmunt et al., 1993) where a number of agonists and electrical field stimulation were shown to induce contractions. In a recent study we showed that isolated strips of U&LP from the pig bladder dome can also contract in response to neurokinin A and muscarinic receptor stimulation, the responses to NKA being mediated via NK2 receptors (Sadananda et al., 2008). These tissues from the dome of the bladder also develop spontaneous phasic contractions. This spontaneous activity was observed in all U&LP preparations but was generally absent in detrusor muscle strips, with only 25% of detrusor strips demonstrating any spontaneous contractile activity. Furthermore, in detrusor strips that did display this phenomenon, the rate of spontaneous contractions was significantly slower than that of the U&LP. The activity was insensitive to tetrodotoxin, which makes it unlikely to be neuronally mediated. Whether the spontaneous activity originates in the smooth muscle or from another cell type is not clear. A network of myofibroblasts exists in the lamina propria and it has been suggested that these cells are similar to the Interstitial Cells of Cajal which act as pacemakers of electrical and contractile activity in the gastrointestinal system (Fry et al., 2007, Ikeda et al., 2009, Ward and Sanders, 2001). Electrophysiological studies have shown that the myofibroblasts of the bladder suburothelium may develop spontaneous intracellular calcium and membrane potential transients and can function as a syncitium (Fry et al., 2007). The present results suggest that this cellular activity may be involved, and is potentially translated into contractile activity at an intact tissue level resulting in spontaneous phasic contractions, the frequency of which was increased by carbachol. Of interest is the report that the frequency of transient calcium and membrane potential changes in bladder myofibroblasts is also increased during muscarinic receptor stimulation (Fry et al., 2007). In
some tissues activation of muscarinic receptors stimulates the production of prostaglandins (Hara et al., 2009) or nitric oxide, (Andersson et al., 2008) however the U&LP contractile responses to carbachol were insensitive to indomethacin and L-NNA.

The detrusor smooth muscle possesses a mixed population of M2 and M3 muscarinic receptors, with a M2:M3 ratio of about 3:1 in most species (Yamanishi et al., 2002, Chess-Williams, 2002). However the responses of isolated detrusor strips to muscarinic agonists are mediated via the minor population of M3 receptors (Sellers et al., 2000, Chess-Williams et al., 2001, Fetscher et al., 2002). A similar result was obtained in the urothelium and lamina propria. Molecular biology and radioligand binding studies indicate that M2 receptors predominate at the mRNA and protein level in this tissue, (Mansfield et al., 2005, Bschleipfer et al., 2007) but frequency responses to carbachol were significantly reduced by the M3 selective antagonist 4-DAMP. The M1 selective antagonist pirenzepine had no effect on responses, whilst the M2 selective antagonist methoctramine, had no effect at lower concentrations and even at 1µM only had a minor effect on tension but not frequency responses. This effect of methoctramine on tension responses to carbachol would be due to a lack of selectivity for this antagonist at the higher concentration resulting in M3 receptor antagonism with the 1µM concentration. In contrast, 4-DAMP significantly reduced frequency and tension responses to carbachol at a very low concentration (10nM), indicating the increase in the rate of phasic spontaneous contractions of the U&LP to carbachol was mediated via the M3 receptor subtype.

The relevance of the spontaneous contractile activity is unknown. Detrusor overactivity results from involuntary contractions demonstrated by cystometry during the filling stage where the bladder, including the urothelium, undergoes periods of stretch. Thus it is possible
that the contractile pacemakers identified in the U&LP may be activated during stretch and may possibly then drive contractions of the detrusor. To investigate this possibility, we examined the effects of stretching the tissues and found that the frequency of the phasic contractile activity was increased. This effect could be direct on the cells initiating the phasic contractions, whether smooth muscle, myofibroblasts, or another cell type sensitive to stretch, or the activity might be driven by factors being released from the U&LP during stretch.

To examine the latter hypothesis, a number of factors known to be released from the urothelium during stretch were investigated including ATP, acetylcholine (Ach) and nitric oxide (Birder et al., 2010, Smith et al., 2008). Although NO is usually inhibitory, there is evidence to suggest that it can exert excitatory effects in the bladder (Gillespie and Drake, 2004). Stretching tissues resulted in an increase in the frequency of phasic contractions, but this was not altered in the presence of L-NNA. ATP and Ach are also released during stretch of the U&LP, but when tested on the isolated tissues in the present study, only Ach produced an increase in spontaneous rate (21 ± 4% increase in rate to 10µM Ach, P<0.01; no significant increase to 1mM ATP, n=8 for both). Furthermore, desensitising P2X purinergic receptors with α,β-methyleneATP had no effect on the stretch-induced responses, whereas atropine completely abolished the frequency response to stretch indicating that Ach released from the U&LP was responsible for the stretch-induced increases in spontaneous rate. In support of this, 4-DAMP depressed these responses, whilst methoctramine and pirenzipine failed to affect frequency responses to stretch, again indicating that responses were mediated via the M3 receptor subtype. Thus, stretch appears to induce the release of Ach from the U&LP which then activates M3 receptors on the cells initiating the spontaneous contractile activity.
There is some evidence to suggest that this activity in the U&LP may be able to drive detrusor smooth muscle activity. Firstly, electrophysiological studies in the rat have shown that the calcium and membrane potential transients produced by carbachol begin near the urothelial-suburothelial interface before spreading to the detrusor (Zygmunt et al., 1993). Secondly, when bladder sheets were examined, spontaneous contractions were greater in bladder tissue with an intact urothelium/lamina propria (Sui et al., 2008). Finally, the increased contractions recorded in the bladders of cats with feline cystitis have been shown to be associated with enhanced calcium transients and supersensitivity to muscarinic stimulation in the urothelium/lamina propria (Ikeda et al., 2009). Thus, it appears possible that spontaneously active cells in the lamina propria may be able to drive detrusor contractions, at least in the overactive bladder where gap junctions are increased (Roosen et al., 2009). Future studies will be required to test this hypothesis.

Currently, the main treatments for overactive bladder are muscarinic antagonists. The exact clinical mechanisms of action for these drugs is unclear, however this study has shown that these drugs will antagonise the muscarinic receptors on the cells regulating U&LP contractile activity, thus preventing these actions of Ach released during stretch. Tolterodine, solifenacin, darifenacin and oxybutynin all depressed the increases in U&LP frequency and tension induced by carbachol. Thus, the responses of the U&LP may represent another possible site of action for these clinically effective anti-muscarinic agents.
Conclusions

The urothelium/lamina propria exhibits spontaneous phasic contractile activity which is increased during stretch. The mechanism appears to involve acetylcholine and M3 muscarinic receptors. Anticholinergic drugs used clinically depressed these responses and this mechanism may represent an additional site of action for these drugs in the treatment of bladder overactivity.

Acknowledgements

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Statement of conflicts of Interest

The authors state no conflict of interest.


alterations in urothelial ATP and NO release induced by chronic spinal cord injury. 

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