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Comparative effects of angiotensin II on the contractility of muscularis mucosae and detrusor in the pig urinary bladder

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Abstract

To explore contractile actions of angiotensin II (ATII) on the muscularis mucosae (MM) of the bladder, ATII-induced contractions were compared between MM and the detrusor smooth muscle (DSM) of the pig bladder by isometric tension recordings. Effects of ATII on spontaneous Ca^{2+} transients in MM were visualised using Cal-520 fluorescence. ATII receptor type 1 (ATR1) expression in MM and DSM was also examined by immunohistochemistry. ATII (1 nM – 1 μM) caused phasic contractions of MM in a concentration-dependent manner, while ATII (10 nM – 10 μM) had no or marginal effects on DSM contractility. ATII (100 nM)-induced MM contractions had an amplitude of approximately 70% of carbachol (1 μM)-induced or 90% of U46619 (100 nM)-induced contractions. Candesartan (10 nM), an ATR1 blocker, prevented the contractile effects of ATII (1 nM) in MM, while ATR1 immunofluorescence was greater in MM than DSM. ATII (10 - 100 pM) increased the frequency but not the amplitude of spontaneous Ca^{2+} transients in MM. Both urothelium-intact and -denuded MM strips developed comparable spontaneous phasic contractions, but ATII, carbachol and U46619-induced contractions were significantly larger in urothelium-denuded than urothelium-intact MM strips. In conclusion, the MM appears to have a much greater sensitivity to ATII compared with DSM that could well sense circulating ATII, suggesting that MM may be the predominant target of contractile actions induced by ATII in the bladder while the urothelium appears to inhibit MM contractility.

Keywords: Urinary bladder, muscularis mucosae, angiotensin II, urothelium, detrusor

Abbreviations:

α-SMA, α-smooth muscle actin; DSM, detrusor smooth muscle; MM, muscularis mucosae; PSS, physiological salt solution; SMC, smooth muscle cell; SPC, spontaneous phasic contraction; ATII angiotensin II; ATR1, angiotensin II receptor type 1; OAB, overactive bladder; LUTS, lower urinary tract symptom; RAS, renin-angiotensin system; ACE, angiotensin converting enzyme

1. Introduction

During the storage phase, localised contractions are generated in the bladder wall resulting in a basal tone that allows efficient bladder emptying [1]. Spontaneous 'asynchronous' contractile activity of the bladder is recognised as micromotions arising from spontaneous phasic contractions (SPCs) in individual muscle bundles. Besides the detrusor smooth muscle (DSM) that generates a force during voiding, the contractility of bladder mucosa is increasingly recognised [2]. Muscularis mucosae (MM) is a predominant contractile element in the bladder mucosa [3]. Isolated mucosa strips taken from human bladders develop SPCs [4]. MM contractility is characterised by SPCs that are up to ten times larger compared with those in the DSM [5], while the MM only modestly responds to parasympathetic nerve excitations [3, 6]. Thus, it is likely that the MM plays a significant role during the storage phase.

Increased SPCs in the bladder wall mechanically stimulate mechanosensitive afferent nerves resulting in urinary urgency [7]. Phasic increases in the intravesical pressure, known as non-voiding contractions, have a much larger impact on afferent nerve firing compared with basal line pressure increase [8]. However, the SPCs of the DSM are not associated with detectable increases in intravesical pressure due to the lack of their synchrony amongst multiple contractile units [9]. Thus, it was envisaged that 'latent' activation of mechanosensitive afferent nerves arising from SPCs that cannot be detected by means of intravesical pressure changes, might explain the pathogenesis of overactive bladder (OAB) in patients who do not display increased non-voiding contractions. The MM SPCs may play a substantial role in such pathological conditions considering their anatomical proximity to suburothelial afferent nerves [5].

The systemic renin-angiotensin system (RAS) predominantly functions in cardiovascular and renal regulation, while angiotensin produced by local RAS in many tissues can influence tissue functions in a paracrine manner [10]. In the bladder, angiotensin I is converted to ATII by angiotensin converting enzyme (ACE) and human chymase, a serine protease [11, 12]. Strong reciprocal correlations between local RAS and metabolic syndrome are evident [13], while the causal link of metabolic syndrome with OAB/lower urinary tract symptoms (LUTS) has been suggested [14]. Thus, the roles of ATII in OAB/LUTS has been considered. In human DSM, ATII (1 nM – 10 μM) causes concentration-dependent contractions with maximum contractions equivalent to high KCl (124 mM)-induced contractions [11] but only 40-50% of carbachol (100 μM)-induced contractions [15]. Considering the activation of mechanosensitive afferent nerves by muscle contractions, the role of ATII in MM contractility is of great interest.

In the present study, we compared the contractile actions of ATII on the MM and DSM of the pig bladder and investigated the involvement and expression of angiotensin II receptor type 1 (ATR1) in MM. Effects of low (pM) concentration ATII on spontaneous Ca^{2+} transients in MM were also examined. Additionally, the role of the urothelium in regulating MM contractility was investigated.

2. Materials and Methods

2.1 Tissue preparation

Bladders were obtained from Landrace pigs of both sexes in the local abattoir, and immediately transported to the laboratory (approximately 1 hour) in cold physiological salt solution (PSS) at 4°C. Upon arrival in the laboratory, the bladders were immediately cut opened longitudinally from the top of the dome to the bladder neck. For contractile studies, pieces of full thickness bladder dome (1-1.5 cm square) were isolated and stored in PSS at 4ºC during and before preparation of tissue strips (maximum 12 hours). For immunohistochemistry, smaller pieces (4 mm square) were dissected and immersed in phosphate buffered saline (PBS) at 4°C.

2.2 Contractile studies

To prepare MM strips, the mucosal layer was separated from the DSM and pinned flat in a dissecting dish with the urothelial side facing up. After the partial removal of the urothelial layer, MM bundles were identified under a dissection microscope (see Supp Fig 1). MM strips with the urothelium removed (MM) and MM strips with intact urothelium (MM+U) (2-3 mm×8-10 mm) in which MM bundles predominantly run along their long axis were dissected from adjacent regions. DSM strips (3 mm×10 mm) were also prepared by first removing the mucosal layer, then isolating several DSM layers. Silk threads were tied around the ends of each tissue strip and were mounted under 0.1g resting tension in separate organ baths and perfused with PSS at 36°C gassed with 5% CO_2 in oxygen. Isometric tension changes were recorded and stored digitally on a PC using a Digidata 1400A or 1550B and pClamp 10 software (Axon Instruments).

The tissues (MM, MM+U and DSM) were equilibrated for 1 hour before administering ATII. After washout, the tension was allowed to return to baseline and stable SPCs were generated for 30 minutes. A second response to the muscarinic agonist carbachol (1 µM) was then obtained. After another 30-minute washout period, a third response to the thromboxane A2 agonist U46619 (100 nM) was obtained.

Concentration-response relationships of ATII-induced contractions of MM or DSM were compared in a separate series of experiments. Strips of MM or DSM were prepared from the same bladder and mounted under 1g resting tension in separate organ baths filled with PSS at 36°C gassed with 5% CO_2 in oxygen. The effects of increasing concentrations of ATII were examined in each MM strip (1 nM –

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1 μM) or DSM strip (10 nM – 10 μM). Isometric tension developed was recorded to a PC using a Powerlab system (ADInstruments, Castle Hill, Australia).

In experiments investigating the effects of the AT1-receptor antagonist candesartan, responses of MM to ATII (1 nM) were first obtained and following a washout, tissues were incubated for 30 minutes with candesartan (10 nM). Following this, contractile responses to ATII (1 nM) were again obtained in the presence of the antagonist and after washout, a final response to carbachol (1 μM) was obtained.

2.3 Fluorescence intracellular Ca^{2+} imaging

To visualise intracellular Ca^{2+} dynamics in MM, urothelium-denuded lamina propria preparations were pinned flat on a block of Sylgard (silicone elastomer, Dow Corning Corporation, Midland, Michigan, U.S.A.). Lamina propria preparations were placed urothelial side down, and vascular and connective tissues were carefully removed as much as possible to expose individual MM bundles. To minimize tissue distortion due to muscle contractions, the preparations were stretched using 15-20 tungsten wires (20 μm in diameter). To visualise Ca^{2+} signals in MM cells, after a 1-hour incubation with warmed (36°C) PSS, the preparations were incubated in low Ca^{2+} PSS ($[Ca^{2+}]_o$ = 0.5 mM) containing 10 μM Calbrite520 AM (special packaging, Dojindo, Japan) and cremphor EL (0.01 %, Sigma, St. Louis, MO, USA) for 50-60 minutes at 36 °C. Following incubation, the preparations were superfused with dye-free, warmed (36 °C) PSS at a constant flow rate (about 2 ml/min) for 30 min.

The recording chamber was mounted on the stage of an upright epifluorescence microscope (BX51WI, Olympus) equipped with an electron multiplier CCD camera (C9100, Hamamatsu Photonics) and a high-speed scanning polychromatic light source (C7773, Hamamatsu Photonics). Preparations were viewed with a water–immersion objective (UMPlanFL x10 or x20, Olympus) and illuminated at 495 nm. The fluorescence emission in a rectangular window was measured through a barrier filter above 515 nm, and images were obtained every 100-200 ms (frame interval) with an exposure time of 50-100 ms using a micro photoluminescence measurement system (AQUACOSMOS, Hamamatsu Photonics). Relative amplitude of Ca^{2+} transients was expressed as the ratio (F_t/F_0) of the fluorescence generated by an event (F_t) against baseline (F_0).

2.4 Fluorescence immunohistochemistry

Specimens were embedded in O.C.T. compound (Sakura Finetek, Tokyo, Japan) and frozen in a freezer at -80°C. Sections with a thickness of 14 μm or 30 μm were cut in a cryostat (CM3050 S, Leica

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biosystems, Wetzlar, Germany), mounted on strong adhesive and hydrophilic MAS-coated glass slides (Matsunami Glass Industry, Osaka, Japan) and then fixed in acetone for 15 min at 4 °C.

Bladder sections were immersed in PBS containing 0.3% Triton X-100 for 10 min, immersed in Block Ace for 20 min and then incubated with primary antibodies for 3 days at 4°C. The primary antibodies used were mouse monoclonal antibody for α-smooth muscle actin (α-SMA, 1:200, clone 1A4, Sigma, St. Louis, MO, USA) and rabbit anti-angiotensin II type I receptor (AT1R, 1:100, ab18801, abcam, Cambridge, UK). The sections were incubated with biotinylated swine anti-rabbit IgG antibody (1:300, Dako, Glostrup, Denmark) for 30 min, and then incubated with ALEXA488-conjugated donkey anti-mouse IgG antibody (10 µg/ml, abcam) and Cy3-conjugated streptavidin (4.5 µg/ml, Jackson ImmunoResearch, West Grove, PA, USA) as well as the nuclear staining reagent Hoechst 33342 (10 µg/ml, Molecular Probes). Specimens were coverslipped with fluorescent mounting medium (Dako, Glostrup, Denmark) and observed using a confocal laser scanning microscope (FV3000, Olympus, Tokyo, Japan).

2.5 Solutions

The composition of PSS was (mM): NaCl 137.4; KCl 5.9; $MgCl_2$ 1.2; $CaCl_2$ 2.5; $NaHCO_3$ 15.5; KH_2PO_4 1.2 and glucose 11.5. Drugs used were human ATII, candesartan, carbachol and U46619 (Sigma and Wako Pure Chemical Industries, Osaka, Japan). Candesartan and U46619 were dissolved in 100% ethanol, and other drugs were dissolved in the distilled water. The final volume in PSS did not exceed 1:1000 for the distilled water and 1:10000 for ethanol.

2.6 Calculations and statistics

For spontaneous phasic contractions (SPCs) and Ca^{2+} transients, the following parameters were measured; peak amplitude, defined as an average of the values measured from the basal tension to the peak of SPCs; half width, measured as the time between 50% peak amplitude on the rising and falling phases; frequency, which was defined as an average over a 3-minute recording of the events. The amplitude of agonist-induced contractions was also measured. Values were expressed as mean ± SD, where 'n' represents the number of preparations from a minimum of 6 animals. The events were captured and averaged using the template search and averaging function of Clampfit 10 software (Molecular Device). This descriptive study comparing responses of the tissues to ATII employed Students' *t*-tests (where two groups were compared) or one-way or two-way ANOVA (where more than two groups were compared) with $p < 0.05$ being considered statistically significant.

3. Results

3.1 ATII-induced contractions of MM and DSM

Strips of MM developed SPCs and bath-applied ATII (100 nM) caused an increase in basal tension that reached its peak and gradually declined in continued exposure to ATII (Fig.1A). Following washout, subsequent ATII (100nM) application failed to generate reproducible contractions and was not sufficient to prevent this tachyphylaxis effect. To minimise the influence of ATII receptor tachyphylaxis on subsequent responses, ATII was removed immediately after inducing peak contractions in the tissue strips.

In spontaneously active MM strips, ATII (100nM) induced a transient contraction and increased SPCs (Fig.1B). Application of muscarinic receptor agonist carbachol (1 μM) induced biphasic contractions consisting of an initial phasic phase followed by a relatively sustained contraction (Fig.1C) while thromboxane A2 agonist U46619 (100 nM) caused sustained contractions (Fig.1D). The magnitude of ATII-induced contractions was approximately 70% and 90% relative to carbachol (Fig.1H) and U46619 (Fig.1I) induced peak contractions, respectively.

In DSM strips, ATII (100 nM) caused only marginal contractions (Fig.1E), compared to carbachol (1 μM, Fig.1F) and U46619 (100 nM, Fig.1G) which caused larger peak contractions. The magnitude of ATII-induced contractions was less than 5% of carbachol (Fig.1H) or U46619 (Fig.1I) induced peak contractions. Comparisons in absolute values of carbachol-induced contractions indicates that DSM strips developed 3 times greater muscle force than MM strips (Fig.1J).

In MM strips, ATII (1 nM – 1 μM) caused contractions in a concentration-dependent manner that reached maximum values of approximately 50% of carbachol(1 μM)-induced contractions at 100 nM (Fig.1K). In contrast, ATII (10 nM – 10 μM) caused no or marginal contractions in DSM, and high concentration of ATII (10 μM) induced less than 10% of carbachol(1 μM)-induced contractions (Fig. 1K).

3.2 Involvement of ATR1 in ATII-induced MM contractions

Because of the ATII-induced tachyphylaxis at 100 nM, reproducibility of a lower concentration of ATII (1 nM)-induced contractions were examined. ATII (1 nM) increased the frequency of SPCs (Figs.2B-C) and the basal tension (Fig.2A) in MM strips. Unlike higher concentrations of ATII, ATII (1 nM) developed sustained contractions for at least 10 minutes of application. Washout periods of ATII greater

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than 30 minutes resulted in MM contractions that were similar to the preceding ATII-induced contractions (Fig. 2B).

The effects of ATII receptor type 1 (ATR1) blocker candesartan on ATII (1 nM)-induced contractions were examined. In MM strips in which ATII (1 nM) induced a sustained contraction, candesartan (10 nM) was applied prior to a second ATII application. In the presence of candesartan, the second application of ATII (1 nM) failed to cause either increases in SPC frequency (Fig.2C) or raise the basal tension (Fig.2A). During continuous exposure to candesartan with ATII, SPCs were present and subsequent application of carbachol (1 μM) developed large contractions comparable to control conditions, indicating that candesartan specifically inhibited ATII-induced contractions.

3.3 Expression of ATR1 in ATII-induced MM contractions

Double immunohistochemistry for ATR1 and α-smooth muscle actin (α-SMA) visualised the distribution of ATR1 in α-SMA positive- and -negative elements within the pig bladder wall. AT1R immunoreactivity was detected in α-SMA-immunoreactive MM and vascular smooth muscle (VSM) of mucosal vasculature (Fig.3A-C). The ATR1 signals in MM and VSM were not found in the negative control using neighbouring sections (Fig.3D), confirming the specificity of the ATR1 antibody. However, ATR1 immunoreactivity was evident in the urothelium (Fig.3A-C) of negative control specimen (Fig.3D), and thus it was not possible to examine ATR1 expression in the urothelium.

AT1R immunoreactivity was also detected in α-SMA-immunoreactive DSM (Fig.3E-G), while AT1R immunoreactivity in DSM was not observed in negative control specimens (Fig.3H). Consistent with the results of contractile studies, MM expressed brighter ATR1 immunofluorescence compared with DSM.

3.4 Effects of ATII on spontaneous Ca2+ transients in MM

In MM bundles of the pig bladder, spontaneous Ca^{2+} transients preferentially originated from either of the boundaries of MM bundles and propagated to the other boundary as previously observed in the case of DSM of the guinea-pig bladder [9]. Thus, the propagating Ca^{2+} transients (Fig.4B) developed temporally correlated rises in the Ca^{2+} level across the MM bundles (Fig.4A).

ATII (10 pM) increased the frequency of the Ca^{2+} transients without changing their amplitude or half-width, while a lower concentration of ATII (1 pM) had no effect. However, ATII (100 pM) further

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increased the frequency and spontaneous Ca^{2+} transients and prolonged their half-width (Figs.4C-D).

3.5 Role of the urothelium in regulating MM contractility

MM strips with intact urothelium (MM+U) exhibited SPCs similar to that seen in MM strips without urothelium. Neither the amplitude (Fig.5A), half width (Fig.5B) nor frequency (Fig.5C) of SPCs were significantly different in MM+U strips compared with the MM strips. ATII (100 nM) caused transient contractions in MM+U strips (Fig.5D) while carbachol (1 μM) and U46619 (100 nM) caused sustained contractions in these strips (Fig.5E-F). The amplitude of ATII, carbachol and U46619-induced contractions were significantly smaller than the corresponding contractions in MM strips without urothelium (Fig.5G-H).

4. Discussion

The DSM has been shown to possess enzymes that generate ATII, and receptors for this peptide which when activated induce contractile responses [12, 16-19]. Angiotensin receptors have been identified on the arteries in the submucosa, but our study show that ATR1 are also present in the mucosa on the MM. Furthermore, the receptors are functional, increasing basal tonic tension and accelerating phasic contractile activity. These responses are elicited at low concentrations of ATII (as low as pM range) and therefore relevant physiologically where circulating ATII concentration has been reported to be at 10 - 50pM [20, 21]. Local RAS has been demonstrated in animal and human tissues and can greatly increase local ATII concentration [11, 17]. Thus, the 10pM and 100pM concentrations in the current study are within physiological levels [11, 15].

RAS is involved in many pathological conditions that have a causal link with LUTS/OAB. Besides the critical roles of RAS in hypertension, its associations with metabolic syndrome and diabetes mellitus are recognised [13]. Serum ACE levels are elevated in several pathological conditions including diabetes mellitus with renal failure [22]. Whether local ATII synthesis within the bladder is altered in conditions such as OAB is currently unclear. However, angiotensin receptor blockers and ACE inhibitors have been shown to reduce bladder dysfunction in several animal models of bladder overactivity [18, 23]. Since the contractility of MM is enhanced by pM range of ATII, MM can readily sense circadian rhythms of RAS or its upregulation in pathological conditions. Thus, MM appears to play an important role in normal bladder function and in bladder pathology in association with RAS.

The results from our functional antagonist study suggest that the responses of the porcine MM to ATII are mediated via the ATR1 subtype. This receptor has been previously identified in the DSM of the rat, rabbit and human bladder [11, 15, 18, 19], but its effect has never previously been examined in the MM. Our results show that the greatest responses to ATII occur in the MM and not the detrusor, with MM contractions being 10-fold greater than DSM responses. This suggests that the physiological role of angiotensin in the bladder may involve the MM more than the DSM. The MM, with its spontaneous and stimulated phasic contractile activity is the nearest contractile element to the sensory nerves, which are known to be activated by mechanical activity [8]. MM may therefore sensitize afferent nerves during bladder filling and ATII could enhance this effect.

The MM is in close proximity to the urothelium which is known to release factors that influence afferent nerve activity and inhibit contraction of the DSM. The presence of the urothelium in intact

tissue strips of porcine bladder wall reduces detrusor carbachol-induced contractions attributed to the release of a diffusible unidentified factor [24]. We examined the responses of MM to ATII, carbachol and a thromboxane agonist and found the presence of the urothelium inhibited these agonist-induced contractions, suggesting that ATII and thromboxane A2 also induces the release of this factor and that MM is sensitive to it. In this study, we were unable to determine whether the urothelium expresses ATR1 and therefore acknowledge that ATII might stimulate release of mediating factors that contribute to MM contractility.

Another observation was the desensitization of contractility to ATII in its continued presence. Repeated administration of ATII after a previous administration failed to initiate contractions. Tachyphylaxis has been reported for ATII-induced responses of isolated human DSM [11, 15], but we are first to demonstrate this phenomenon in the MM. The cellular mechanism underlying this phenomenon was not investigated in this study but previous studies have reported AT1R activation causing rapid internalization of receptors [25]. It is likely that this mechanism might operate in the bladder, regulating the sensitivity of the MM to ATII.

5. Conclusions

The MM appears to be the main target for ATII at concentrations as low as pM ranges, inducing tonic contraction and increasing phasic contractile activity. Due to the close proximity of afferent nerves to the MM, we hypothesise that this phasic activity could potentially trigger afferent activity and micturition, but requires further investigations using nerve recording. The response of the MM to ATII are mediated via the AT1R subtype and inhibited in the presence of the urothelium. This study identifies the main target for ATII actions in the porcine bladder and suggest a sensory mechanism for its effects on micturition.

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Compliance with Ethical Statements

Declaration of Interest: On behalf of all authors, the corresponding author states that there is no conflict of interest.

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Figure Legends

Fig. 1 *Comparative effects of ATII on MM and DSM*

(A) In a MM strip of the pig bladder that developed SPCs, prolonged application of ATII (100 nM) resulted in a gradual decline in contraction after achieving the peak contraction. (B) In another MM strip, ATII (100 nM) induced a transient contraction. In the same strip, (C) CCh (1 µM) and (D) U46619 (100 nM) induced a sustained contraction. (E) In a DSM strip of the pig bladder, ATII (100 nM) induced small oscillatory contractions. In the same strip, (F) CCh (1 µM) or (G) U46619 (100 nM) caused a sustained contraction. The relative amplitude of ATII-induced contractions (%) against (H) CCh (MM: n=15, DSM: n=21, *p<0.05, unpaired *t*-test, two-tailed) or (I) U46619 (MM: n=17, DSM: n=20, *p<0.05 unpaired *t*-test, two-tailed) was summarised. (J) The mean amplitude of CCh-induced contractions was approximately three times larger in DSM strips compared to MM strips (MM: n=15, DSM: n=21, *p<0.05, unpaired *t*-test, two-tailed). (K) In MM strips, ATII (1 nM – 1 µM) caused contractions in a dose-dependent manner *(blue)* that reached approximately 50% of CCh(1 µM)-induced contractions. In DSM strips, ATII (10 nM – 10 µM) caused much smaller contractions *(red)* compared with ATII-induced contractions with their maximum value of less than 10% of CCh (1 µM)-induced contractions (n=6, *p<0.05, ***p<0.001, unpaired *t*-tests, two-tailed).

Fig. 2 *Effects of candesartan on ATII-induced MM contractions*

(A) In a MM strip that developed SPCs, the first application of ATII (1 nM) increased their frequency with a rise in the basal tension. In the same strip that had been treated with candesartan (10 nM) for 25 minutes, the second application of ATII (1 nM) failed to cause changes in SPCs or the baseline tension. Subsequent addition of CCh (1 µM) caused a large, sustained contraction. (B) In another MM strip, 30 minutes following washout of the first ATII (1 nM) application, a subsequent application of ATII (1 nM) was capable of reproducing contractions that had an equivalent amplitude to those of the first. (C) The mean amplitude of MM contractions induced by first and second applications of ATII (1 nM) in control conditions were summarised (n=7, paired *t*-test, two-tailed) (D) In control conditions, the second ATII (1 nM)induced response increased the frequency of SPCs by approximately 100% (n=7, *p<0.05, paired *t*-test, two-tailed). In MM strips that had been treated with candesartan (10 nM), the second ATII (1 nM) application failed to increase SPC frequency (n=7, paired *t*-test, two-tailed).

Fig. 3 *Expression of AT1R in MM and DSM*

Sections of pig bladder were immunostained with α-smooth actin (green), AT1R antibodies (red) and nuclear stain Hoechst 33342 (blue) to show mucosal structure. The α-smooth actin (green) was

observed in the MM, vascular smooth muscle (VSM), and DSM (A, B, E and F). The MM and VSM expressed AT1R (C and G). When the AT1R antibody was omitted, no signal was detected in the MM (D and H), whilst the urothelium showed non-specific reaction (D).

Fig. 4 *Effects of ATII on spontaneous Ca^{2+} transients in MM*

(A) In a MM bundle of the pig bladder where spontaneous Ca^{2+} transients were recorded from three ROI, ATII (10 pM) increased the frequency of the Ca^{2+} transients. (B) Overlaid traces with an expanded time scale demonstrated that spontaneous Ca^{2+} transients propagated across the MM bundle. Effects of ATII (1-100 pM) on the amplitude (C), half-width (D) and frequency (E) of spontaneous Ca^{2+} transients were summarised (control: n=17, 1pM: n=5, 10pM: n=5, 100pM: n=7, *p<0.05, one-way ANOVA followed by Bonferroni post-hoc test).

Fig. 5 *Comparison of spontaneous and ATII-induced contractions between the urothelium intact- and denuded-MM*

In urothelium-intact (UC(+)) MM strips, the (A) amplitude, (B) frequency and (C) half width of SPCs were not significantly different from those of urothelium-denuded (UC(-)) MM strips (UC(+): n=14, UC(-): n=15, unpaired *t*-test, two-tailed). (D) In a urothelium-intact (UC(+)) MM strip that developed SPCs, ATII (100 nM) induced a sustained contraction. In the same strip, (E) CCh (1 µM) or (F) U46619 (100 nM) caused a larger sustained contraction. (G) ATII (100nM), (H) CCh (1µM) and (I) U46619 (100nM) induced smaller contractions compared to those in UC(-) strips (UC(+): n=14, UC(-): n=15, *p<0.05, unpaired *t*-tests, two-tailed).

Supp Fig. 1: *Urothelium-intact and denuded MM strip dissection*

(A) Urothelium (UC)-intact (UC (+)) MM strips immunostained with α-smooth muscle actin (α-SMA, *red*), while nuclei were stained using Hoechst 33342 (*blue*). Submucosal blood vessels (BV) are located in the connective tissue layer between muscularis mucosae (MM) and detrusor smooth muscle (removed). See Figure 1A in Mitsui et al. (2020), reference no. 3. (B) UC-denuded (UC (-)) MM strips immunostained with α-SMA *(red)* and Hoechst 33342 (*blue*). (C) Photo of isolated MM bundles identified under the dissection microscope. The urothelium (UC) remained attached in the upper third region, while the urothelium was removed in the remaining region. Muscularis mucosae (MM) and suburothelial blood vessels (BV) can be seen in the urothelium-denuded region.