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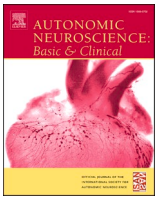

Piezo1, but not ATP, is required for mechanotransduction by bladder mucosal afferents in cystitis

Wai Ping Yew, Timothy Hibberd, Nick J. Spencer, Vladimir Zagorodnyuk *

Discipline of Human Physiology, Flinders Health & Medical Research Institute, College of Medicine and Public Health, Flinders University, South Australia, Australia

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ABSTRACT

Piezo ion channels play a role in bladder sensation, but the sensory afferent subtypes that utilise Piezo channels have not been fully explored. We made single-unit extracellular recordings from mucosal-projecting bladder afferents in guinea pigs with protamine/zymosan-induced cystitis. The Piezo1 agonist, Yoda1, significantly potentiated mechanosensitivity, while its antagonist, Dooku1, abolished this potentiation. The P2 purinoceptor antagonist, PPADS abolished α,β-methylene ATP-induced excitation of mucosal afferents without affecting their mechanical activation or potentiation of mechanosensitivity by Yoda1. The findings suggest Piezo1, but not ATP, is required for mechanotransduction in bladder mucosal afferents in cystitis.

1. Introduction

Two primary mechanisms have been demonstrated to underlie mechanotransduction: (i) a direct transduction mechanism that relies on mechano-gated channels expressed by the primary afferent terminal endings, which open in response to physical membrane distortion to initiate membrane depolarisation and (ii) an indirect transduction mechanism that relies on the release of mediators from mechanosensitive non-neuronal cells, which then activate afferent endings (Hamill and Martinac, 2001).

In the bladder, the current widely discussed hypothesis suggests that urothelium-released ATP is involved in mechanotransduction by activating primary afferent fibres in the bladder mucosa and is thus crucial for bladder nociception and promoting voiding during bladder distension (Burnstock, 2001; Birder and Andersson, 2013). Exogenous ATP is capable of exciting several classes of bladder afferents in mice and guinea pigs (Vlaskovska et al., 2001; Rong et al., 2002; Zagorodnyuk et al., 2007, 2009). However, we have previously failed to confirm a role for ATP as a mechanotransduction molecule for low threshold stretch-sensitive muscular-mucosal afferents and stretch-insensitive mucosal afferents in naïve guinea pig bladders (Zagorodnyuk et al., 2007, 2009). It has been shown that ATP release from the urothelium is augmented in cystitis (Sun et al., 2001; Birder et al., 2010), suggesting a role of ATP in mechanosensory transduction and cystitis-induced pain in patients with interstitial cystitis/bladder pain syndrome (IC/PBS). We recently showed that in a guinea pig model of IC/BPS, mucosal afferents are sensitised via up-regulation of TRPV1 and TRPM8 channels, and their combined antagonism resolves cystitis-induced hyperalgesia (Ramsay et al., 2023). Thus, studying cystitis in guinea pigs can provide a great opportunity to identify the potential contribution of endogenous ATP underlying mechanotransduction of the mucosal afferents as these afferents have their receptive fields in the vicinity of the urothelium (Zagorodnyuk et al., 2007, 2009), and so they are well suited to be responsive to ATP released from the urothelial cells upon their mechanical activation.

The discovery of Piezo1 and Piezo2 cation channels as major mechanosensitive channels in neuronal and non-neuronal cells (Coste et al., 2010; Delmas et al., 2022) prompted numerous studies on their functional role in lower urinary tract organs (Li et al., 2022). Piezo1 cation channels mediate mechanosensitivity in a large variety of non-excitable cells and are activated by various forms of mechanical stimulation, including poking, stretching, shear stress, and fine touch (Coste et al., 2010; Li et al., 2022; Delmas et al., 2022). Piezo1 is widely expressed in detrusor smooth muscle cells, suburothelial and intramuscular interstitial cells, PDGFR-alpha-positive interstitial cells, and all urothelial cells (Michishita et al., 2016; Liu et al., 2018; Dalghi et al., 2021). Stretch-induced activation of Piezo1 in the isolated cultured urothelial cells leads to an increase in $[Ca^{2+}]_i$ followed by ATP release from urothelium (Miyamoto et al., 2014). Piezo1 mechanosensitivity and its abundance in bladder urothelium suggest it may indirectly

* Corresponding author at: Discipline of Human Physiology, College of Medicine and Public Health, Flinders University, GPO Box 2100, Adelaide, South Australia, Australia.
E-mail address: vladimir.zagorodnyuk@flinders.edu.au (V. Zagorodnyuk).

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regulate mucosal afferent excitability by triggering urothelial ATP release onto their receptive endings, as well as a potential direct role via expression of Piezo1 on their receptive endings. Here, we aimed to uncover the roles of Piezo1 and ATP in the mechanical activation of mucosal afferents in animal models of IC/PBS.

2. Materials and methods

This study was approved by the Flinders University Animal Welfare Committee (AEM1574-5) and performed in accordance with the ARRIVE guidelines and the Australian Code for the Care and Use of Animals for Scientific Purposes 8th edition. Adult female guinea pigs (300–400 g, N = 19) were housed in a normal light cycle with ad libitum access to a standard diet and water.

Protamine/zymosan-induced cystitis was described in detail previously (Ramsay et al., 2023). In anaesthetised guinea pigs, a lubricated catheter was introduced via urethra into the bladder. Protamine sulfate (10 mg/mL in PBS) was instilled for 1 h, followed by saline washes and then zymosan A (10 mg/mL in PBS) instillation for 1 h. Guinea pigs were allowed 24 h to recover before experiments.

Ex vivo bladder preparations were described previously (Ramsay et al., 2023). Several trunks entering the bladder trigone region were freed from connective tissue, immersed in liquid paraffin for electrical isolation, and individually placed on a platinum recording electrode. Action potentials were amplified with a DAM80 (WPI, USA) and recorded and digitised at 20 kHz using a Micro 1401-4 data acquisition module (CED-UK). Single units were discriminated in Spike 2 software (v10, CED, UK).

A single class of bladder afferents was investigated in this study — high-responding mucosal afferents. As shown previously, these afferents respond to stroking of their mucosal receptive fields with light von Frey hairs (10–100 mg), but not bladder wall stretch (1–40 g) (Zagorodnyuk et al., 2007; Ramsay et al., 2023). To activate mucosal afferents, their receptive field was stroked at a rate of 5 mm·s^{-1} with a 100 mg calibrated von Frey hair and 3 stroke responses were averaged and used for analysis. Maximal effort was dedicated to ensuring consistent mechanical stimuli were applied to the same receptive area in individual preparations (its borders were marked with carbon particles at the beginning of experiments). To allow appropriate drug penetration, a small hole (2 × 2 mm) was made in the mucosa adjacent to the receptive field. α,β-Methylene ATP (α,β-me ATP) was spritzed on the receptive field area; their responses were averaged for a 10s period around the peak response. Yoda1, Dooku1, PPADS, α,β-me ATP and zymosan A were obtained from Sigma-Aldrich (Australia) while protamine sulfate was from TCI Chemicals (Australia). Drug concentrations used in the study are based on available literature and our previous findings (Zagorodnyuk et al., 2007, 2009; Evans et al., 2018; Beca et al., 2021).

3. Results

We have recently characterised protamine/zymosan-induced cystitis as an appropriate animal model of the common non-ulcerative form of IC/BPS; protamine/zymosan treatment induces mild inflammation, hyperreflexia and hyperalgesia in female guinea pigs. The effects of protamine/zymosan-cystitis on spontaneous activity and mechanosensitivity of mucosal bladder afferents have also been previously described in detail (Ramsay et al., 2023). In this study, we focused on the mechanosensitivity of mucosal bladder afferents in guinea pigs with cystitis.

In protamine/zymosan inflamed bladders, von Frey hair (100 mg)

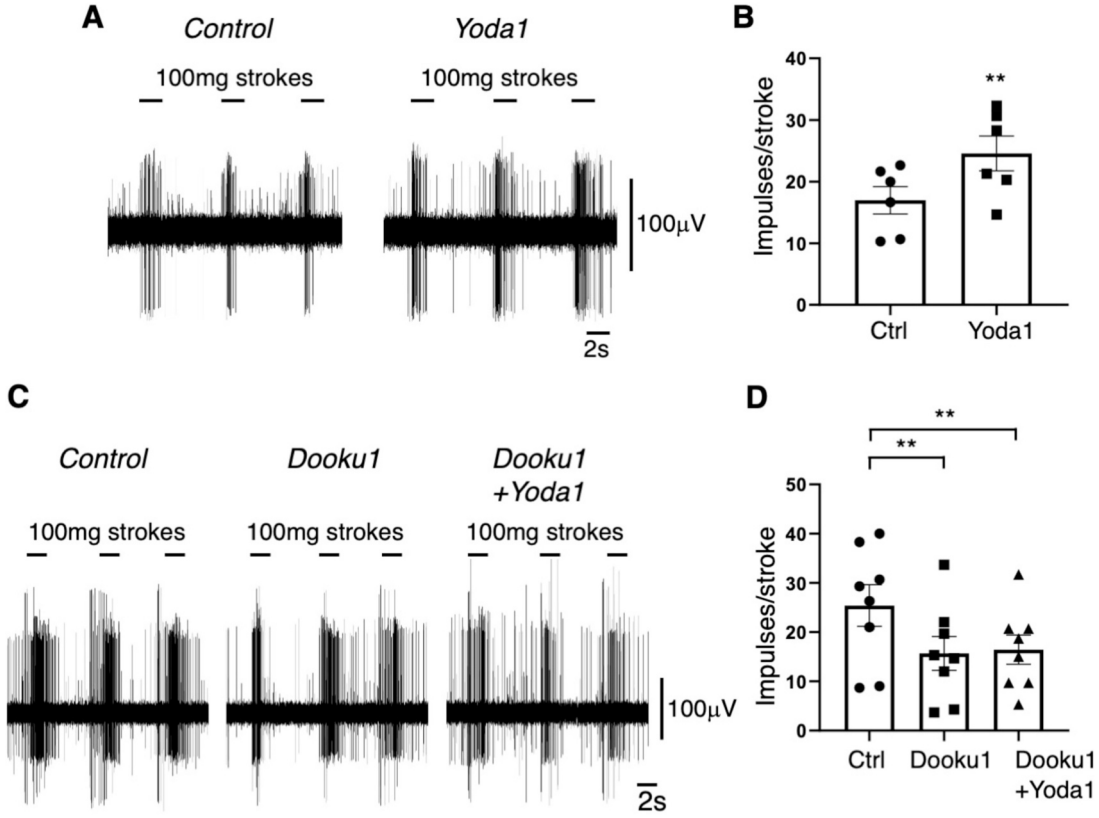

Fig. 1. The effect of Yoda1 and Dooku1 on mechanosensitivity of mucosal afferents.
(A) Raw traces of the responses of mucosal afferents to von Frey hair stoking (100 mg) in the absence and presence of Piezo1 agonist Yoda1 (20 μM). (B) Group data of potentiation of bladder mucosal afferents by Yoda1 (20 μM). (C) Raw traces of the responses of mucosal afferents to von Frey hair stoking (100 mg) in the absence and presence of Dooku1 and Dooku1 plus Yoda1. (D) Group data of inhibition of bladder mucosal afferent responses by Dooku1 (10 μM) and Dooku (10 μM) plus Yoda1 (20 μM). Data is presented as the mean ± the SEM; n = 6, N = 4 per group for A, and n = 8, N = 4 for D. $^{**}P < 0.01$.

stroking responses of mucosal afferents were significantly increased by Piezo1 agonist, Yoda1 (Syeda et al., 2015) (20 μM for 10 min) from control 17.0 ± 2.2 impulses/stroke to 24.6 ± 2.8 impulses/stroke (n = 6, N = 4, $P < 0.01$) (Fig. 1A, B), but spontaneous firing was not significantly affected (from 0.5 ± 0.3 Hz to 1.1 ± 0.7 Hz, n = 6, N = 4, $P = 0.16$). Conversely, the Yoda1 inhibitor, Dooku1 (Evans et al., 2018) (10 μM for 20 min) significantly decreased 100 mg von Frey hair stroking responses from control 25.4 ± 4.2 impulses/stroke to 15.7 ± 3.2 impulses/stroke (n = 8, N = 4, $P < 0.01$) (Fig. 1C, D), while spontaneous activity was not changed: from 1.1 ± 0.3 Hz to 1.1 ± 0.4 Hz after 20 min of Dooku1 (n = 8, N = 4, $P = 0.21$). Importantly, Dooku1 (10 μM) blocked the increased mechanosensitive response of mucosal afferents by Yoda1 (to 16.4 ± 3.0 impulses/stroke, n = 8, N = 4) (Fig. 1C, D).

Non-selective P2 purinoceptor antagonist, PPADS (30 μM for 20 min) did not affect responses to 100 mg von Frey hair stroking (20.3 ± 3.1 impulses/stroke versus 19.1 ± 2.8 impulses/stroke, n = 6, N = 6, $P = 0.6$). Subsequent addition of Yoda 1 (20 μM) in PPADS significantly increased mechanosensitivity of mucosal afferents, reaching 25.4 ± 4.2 impulses/stroke (n = 6, N = 6, $P < 0.05$) (Fig. 2A, B). Spontaneous mucosal afferent firing was not affected by PPADS (30 μM for 20 min): 0.5 ± 0.2 Hz versus 0.6 ± 0.2 Hz (n = 6, N = 6, $P = 0.39$) nor by Yoda1 in PPADS (1.0 ± 0.3 Hz, n = 6, N = 6, $P = 0.13$).

P2 purinoceptor agonist, α,β-me ATP (1 mM) spritzed on mucosal afferent receptive field evoked an average firing response of 11.6 ± 2.8 Hz (n = 5, N = 5). Pre-treatment with non-selective P2 purinoceptor antagonist, PPADS (30 μM for 20 min) nearly abolished α,β-me ATP (1 mM)-induced firing (0.2 ± 0.1 Hz, n = 5, N = 5, $P < 0.01$) (Fig. 2C, D).

4. Discussion

The present results reveal that Piezo1 ion channels play an important role in mechanotransduction of mucosal projecting bladder afferents in cystitis. This is supported by the finding that the selective activator of the Piezo1 channels Yoda1 significantly potentiated their mechanosensitivity, while Dooku1 (which is a competitive inhibitor of Yoda1) abolishes this potentiation. To the best of our knowledge, this is the first functional study that directly demonstrates the involvement of Piezo1 in bladder mucosal afferent mechanotransduction.

In bladder preparations ex vivo, at least five major functional types of bladder afferents have been identified: (i) muscular, (ii) mucosal, (iii) muscular-mucosal, (iv) vascular (=serosal), and (v) silent afferents (Christie et al., 2021). Mucosal afferents are low-threshold mechanoreceptors that can be stimulated by light von Frey hair stroking of their receptive fields in the mucosa, but not by stretch (Zagorodnyuk et al., 2007; Xu and Gebhart, 2008).

Urothelial cells were identified as the primary transducers of physical (stretch) and chemical stimuli that transmit this information to closely associated afferent fibre endings, suburothelial interstitial cells and immune cells by releasing a variety of transmitters, including ATP, acetylcholine, neurotrophins, nitric oxide, SP and prostaglandins (Birder and Andersson, 2013). This study implicates Piezo1 in the mechanotransduction mechanisms of bladder mucosal afferents and suggests that ATP is not required for this process. It remains possible that other non-purinergic urothelial transmitters are involved in an indirect mechanotransduction mechanism of mucosal afferents, including those involving Piezo1. Piezo1 channels are expressed in urothelial cells, and their activation can lead to the release of neurotransmitters, including ATP, from the urothelium (Miyamoto et al., 2014; Liu et al., 2018; Dalghi et al., 2021). The converse possibility is that mechanotransduction by mucosal afferents is direct and involves Piezo1 channels present on their terminal endings. Indeed, Piezo1 is expressed in dorsal root ganglia (DRG) neurons, where it can be involved in mechanical itch and mechano-nociception (Wang et al., 2019; Hill et al., 2022). This question warrants further detailed investigation.

The remaining responses to mechanical stimulation of mucosal afferents seen in the presence of Dooku1 could be due to the activation of Piezo2 channels. In the bladder, Piezo2 is found predominantly in sensory fibres but is also expressed in a small subset of superficial umbrella

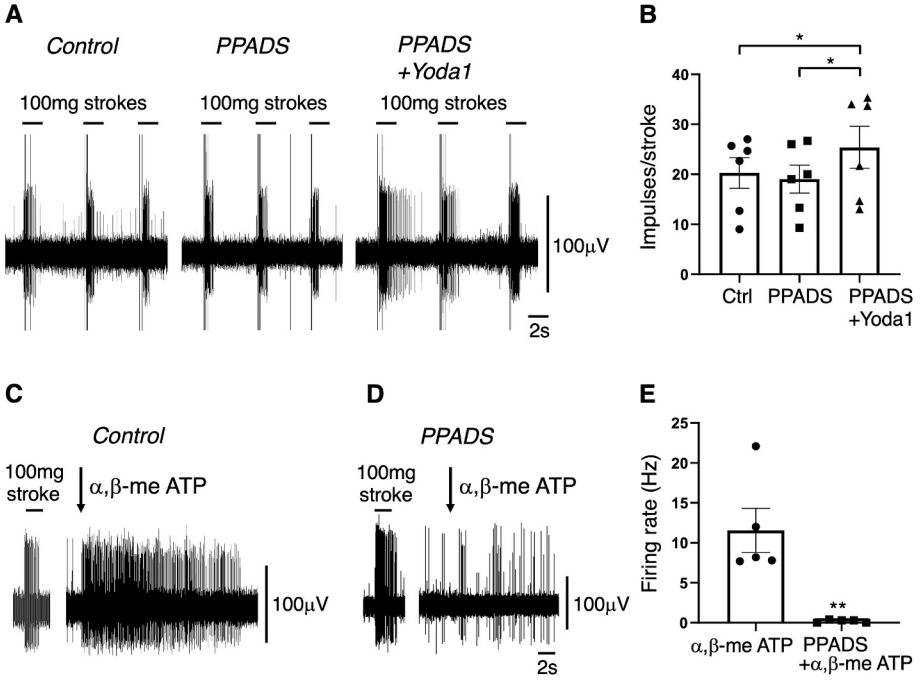

Fig. 2. The effect of PPADS and α,β-me ATP on mechanosensitivity and excitability of mucosal afferents. (A) Raw traces of the responses of mucosal afferents to von Frey hair stoking (100 mg) in the absence and presence of PPADS (30 μM) and PPADS (30 μM) plus Yoda1 (20 μM). (B) Group data of potentiation of bladder mucosal afferents by Yoda1 (20 μM) in the presence of PPADS (30 μM). (C) Raw traces of the responses of mucosal afferents to von Frey hair stoking (100 mg) and administration of αβ-me ATP (1 mM). (D) Raw traces of the responses of mucosal afferents to von Frey hair stoking (100 mg) and administration of α,β-me ATP (1 mM) in the presence of PPADS (30 μM). (E) Group data of inhibition of bladder mucosal afferent responses to α,β-me ATP (1 mM) by PPADS (30 μM). Data is presented as the mean ± the SEM; n = 6, N = 6 per group for B, and n = 5, N = 5 for E. *$P < 0.05$ and **$P < 0.01$.

cells in the urothelium (Marshall et al., 2020; Dalghi et al., 2021). Given the structural similarities between Dooku1 and Yoda1 (Evans et al., 2018), Dooku1 likely acts as a competitive inhibitor of Yoda1, being a "silent binder" with no direct effects on Piezo1 channel kinetics and opening probability (Evans et al., 2018; Wijerathe et al., 2022). Dooku1 abolished potentiating effects of Yoda1 on mucosal mechanosensitivity, which is consistent with the idea that it is a competitive inhibitor of Yoda1. The revealed inhibitory effect of Dooku1 itself on the responses of mucosal afferents to mechanical stimulation is an interesting finding, raising the question of yet undiscovered endogenous activators of Piezo1 at the Yoda1 binding site. In a few experiments on the naïve bladders, we saw similar effects of Yoda1 and Dooku1 on mucosal bladder afferents' mechanosensitivity (data is not shown). This may argue against the possibility that the Dooku1 inhibitory effect is inflammation specific. It is worth mentioning that in the red blood cell membrane, Dooku1, in addition to inhibiting a Yoda1-stimulating effect on Piezo1 channels, activates some cation channels which can be blocked by GsMTx4 (Hatem et al., 2023). This indicates that Dooku1 may have some still unknown effects that warrant future detailed studies of its mechanism of action on the bladder DRG neurons.

The P2 purinoceptor antagonist, PPADS, dramatically reduced α,β-me ATP-induced excitation of mucosal afferents but not their responses to von Frey mechanical stimulation or their potentiation by Yoda1, suggesting that ATP is not involved in the mechanical activation of mucosal afferents in cystitis. This confirms our previous data on naïve guinea pig bladders indicated that PPADS (30 μM) does not affect the mechanosensitivity of the mucosal afferents (Zagorodnyuk et al., 2009). Since we were not measuring the release of neurotransmitters, we cannot completely exclude the possibility that our mechanical stimulus (von Frey stroking of superficial mucosa layer) may not be strong enough to activate urothelial cells (or other non-neuronal cells). In this case, inhibiting P2 purinoreceptors will block ATP response but not mechanotransduction due to the release of endogenous purinergic neurotransmitter from the urothelium. This, however, is unlikely since we previously showed that the stretch- and stroking-induced responses of muscular-mucosal afferents in naïve bladders were also not affected by PPADS while their ATP activation was abolished (Zagorodnyuk et al., 2009).

The mucosal afferents studied here are peptidergic and capsaicin-sensitive and likely participate in signalling of nociceptive information from the bladder (Zagorodnyuk et al., 2010; Ramsay et al., 2023). Because they are not stretch-sensitive, their role in initiating the micturition reflex is minimal, if any. We recently demonstrated several bladder afferents classes were sensitised in cystitis, contributing to visceromotor pain responses; in addition to mucosal afferents, high-threshold muscular and muscular-mucosal stretch-sensitive afferents were likely involved (Ramsay et al., 2023). It was previously shown that purinoreceptor antagonists (non-selective P2X/P2Y antagonists suramin and PPADS, and the P2X1, P2X3 and P2X2/3 receptor antagonist TNP-ATP) reduce distension-induced firing of pelvic high threshold bladder afferents in both multiunit and single-unit recordings (Vlaskovska et al., 2001; Rong et al., 2002). This indicates that ATP may play a mediatory or modulatory role in mechanotransduction by high-threshold stretch-sensitive bladder afferents rather than mucosal afferents. The current widely discussed hypothesis suggests that urothelium-released ATP is critical for bladder nociception (Burnstock, 2001; Birder and Andersson, 2013). The potential contribution of ATP, Piezo1 and presumed nociceptive mucosal afferents to bladder pain needs to be addressed in future in vivo experiments.

5. Conclusions

Piezo1 is likely involved in mucosal bladder afferent mechanotransduction in the guinea pigs with cystitis since Piezo1 activation by Yoda1 potentiated their mechanosensitivity, which was blocked by Dooku1. PPADS abolished α,β-me ATP-induced excitation of mucosal afferents but not von Frey hair stroking-induced responses or their potentiation by Yoda1. These findings argue against the role of ATP as an essential mediator of mechanotransduction by mucosal afferents in cystitis. Stretch-evoked release of neurotransmitters from the urothelium could be involved in urothelial modulation of excitability of different types of bladder afferents, thus contributing additional resolution to bladder mechanosensation in normal physiological conditions and pathological conditions such as overactive bladder and IC/BPS. However, the exact mechanism regulating urothelial-mediated alterations in bladder mechanosensation in healthy bladders and cystitis still needs further exploration since ATP release alone cannot fully explain mechanical activation of mucosal afferents in cystitis.

CRediT authorship contribution statement

Wai Ping Yew: Formal analysis, Data curation. **Timothy Hibberd:** Writing – review & editing, Investigation. **Nick J. Spencer:** Writing – review & editing. **Vladimir Zagorodnyuk:** Writing – review & editing, Writing – original draft, Investigation, Conceptualization.

Informed consent statement

Not applicable.

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Declaration of competing interest

The authors of "Piezo1, but not ATP, is involved in mechanotransduction by bladder mucosal afferents in cystitis" declare no conflicts of interest.

Data availability

Data will be made available on request.

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