

CONNEXINS 43 AND 45 HEMICHANNELS AND CALHM1 MEDIATE ATP RELEASE IN HUMAN UROTHELIAL RT4 CELLS

Hypothesis / aims of study

Over the last two decades, a prominent role of ATP in bladder sensation via the activation of purinergic receptors on sensory afferent neurons to regulate urinary bladder functions under normal and pathophysiological conditions has been established. A significant rise in stretch-induced ATP release from urothelium has been reported in patients with overactive bladder and interstitial cystitis [1]. Although in animal models, such as rat, mice and porcine, ATP release from the bladder has been demonstrated to occur via many different mechanisms, including connexin (Cx)43 and Cx45 hemichannels [2] and pannexin-1 and calcium homeostasis modulator 1 (CALHM1) channels [3], the cellular pathways responsible for ATP release from human bladder are still unknown. Characterising mechanisms involved in ATP release in the human bladder could be the first critical step to develop new therapeutics for disorders associated with increased ATP release. We, therefore, aimed to determine the involvement of pannexin-1, CALHM1, Cx43 and Cx45 in regulating extracellular ATP signalling in human urothelial cells. We hypothesised that pannexin-1 and CALHM1 channels and connexin hemichannels are ATP release channels in the human bladder and that bladder diseases are associated with abnormal expression and function of these channels.

Study design, materials and methods

Immunohistochemistry was conducted on human urothelial RT4 cells using primary antibodies against Cx43 (C6219 Sigma-Aldrich) and Cx45 (ab150077 Abcam), pannexin-1 (ab124131 Abcam) and CALHM1 (HPA018317 Sigma-Aldrich), and a secondary Alexa Fluor488 antibody (ab150077 Abcam) for fluorescent staining. Double labelling has also been performed with the cytokeratin marker AE1/AE3 (M3515 Dako). Cx43, Cx45, pannexin-1 and CALHM1 mediated ATP release in response to hypotonic (~50%) solution induced stretch and extracellular Ca²⁺ depletion ([Ca²⁺]_o, ~17nM) was measured in the presence and absence of channel inhibitors by ATP Bioluminescence Assay (Sigma-Aldrich).

Results

Intensive Cx43, Cx45, pannexin-1 and CALHM1 immunoreactive signals were observed on urothelial cells with a similar expression pattern. Their cellular expression appeared to be dense on cell membranes (Figure 1). Depending on the stage of cell division, the immunoreactive signals could also be observed throughout the cytoplasm, nuclear membranes and inside the nucleus. Double labelling with the epithelial cell marker AE1/AE3 has not only confirmed the urothelial cell identity of RT4 cells, but also demonstrated the presence of these channels on urothelial cells.

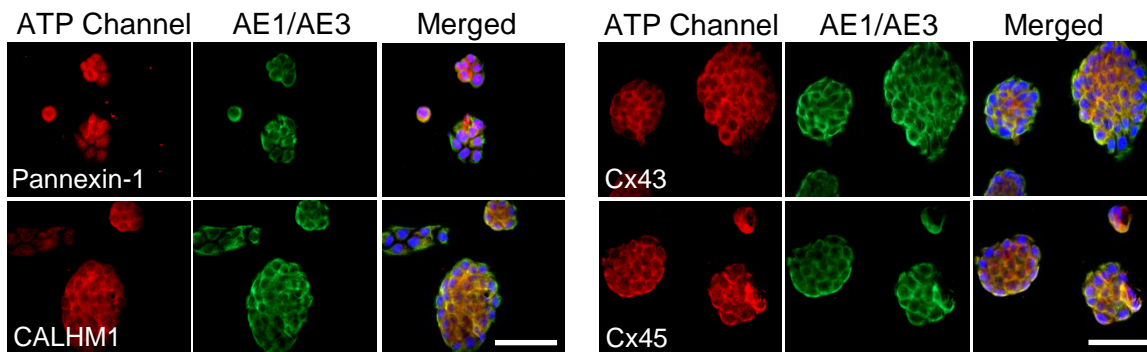


Figure 1: Double labelling of pannexin-1, CALHM1, Cx43 and Cx45 antibodies with the epithelial cytokeratin marker AE1/AE3 antibody in human RT4 urothelial cells.

Functional studies have shown that 50% hypotonic solution which mimics mechanical stretch of the bladder induced a significant rise in ATP release from RT4 cells (Figure 2A). Peptide 5 (20 μ M), a Cx43 hemichannel blocker; a Cx45 mimetic peptide (amino acids 202–217, QVHPFYVCSRLPCPHK), which blocks Cx45 channel formation and ruthenium red (20 μ M), a CALHM1 blocker, significantly reduced the stretch mediated ATP release from urothelial RT4 cells. On the other hand, a trend of decrease of extracellular ATP release was observed in the presence of ¹⁰Panx1 (100 μ M), a selective pannexin-1 channel blocker, but the result was not statistically significant.

Similar to those reported in porcine bladder urothelial cells [2,3], an extracellular Ca²⁺ free ([Ca²⁺]_o) condition significantly stimulated ATP release from RT4 cells, which was significantly inhibited by Peptide 5, Cx45 mimetic peptide and ruthenium red. ¹⁰Panx1 showed no effect on [Ca²⁺]_o potentiated ATP release (Figure 2B).

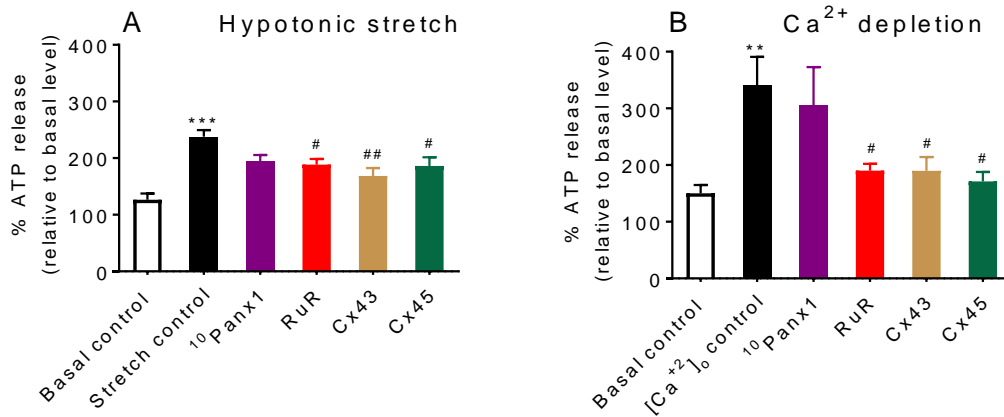


Figure 2: ATP release in response to hypotonic induced stretch (A) and extracellular Ca²⁺ depletion (B). Data were analysed by one-way ANOVA followed by Dunnett test. *P<0.05, **P<0.01, ***P<0.001 compared to basal control; #P<0.05 and ##P<0.01, compared to stretch control or [Ca²⁺]_o control.

Interpretation of results

Intensive Cx43, Cx45, pannexin-1 and CALHM1 staining on human urothelial RT4 cells, especially on cell membranes, suggests that these channels are functional channels in human bladder urothelial cells where they regulate the release of small molecules including ATP to extracellular spaces to mediate various cell functions. Furthermore, since Cx43 and Cx45 are gap junction proteins, they are likely to regulate cell-cell communications, which allow millions of cells to coordinate and work synchronously in the urothelial cells.

Both hypotonic induced stretch and extracellular Ca²⁺ depletion enhanced the release of ATP from RT4 cells and the release involved Cx43, Cx45 and CALHM1 channels. These results indicate that Cx43 and Cx45 hemichannels and CALHM1 play a role in mechanosensitive ATP release in human urothelial cells. The slight inhibition of hypotonic stretch induced ATP release by 10Panx1 may indicate that pannexin-1 is less sensitive to mechanical stretch compared to porcine urothelial cells [3]. Reducing extracellular Ca²⁺ from 2.5 mM to 17 nM increased CALHM1 and connexin channel activities, suggesting that the open/close switch of these channels are Ca²⁺-dependent. 10Panx1 did not alter [Ca²⁺]_o-induced ATP release from RT4 cells, suggesting that pannexin-1 is not regulated by extracellular Ca²⁺.

Concluding message

Here, we report for the first time that Cx43, Cx45 and CALHM1 are ATP release channels in response to physiological stretch and [Ca²⁺]_o in the human bladder urothelial cells, suggesting that these channels may play an important role in the initiation of autocrine/paracrine signalling in response to bladder distension during the storage phase of micturition reflex. Many studies have demonstrated an augmented level of extracellular ATP in the urothelium of patients with interstitial cystitis, overactive bladder and urge incontinence [1]. Further studies are required to investigate whether the expression and function of these ATP release channels are exaggerated in these conditions to reveal their role in urinary dysfunction.

References

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Disclosures

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