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Pannexin 1 channels mediate the release of ATP into the lumen of the rat urinary bladder

Jonathan M. Beckel[1,2,5], Stephanie L. Daugherty[2], Pradeep Tyagi[3], Amanda S. Wolf-Johnston[4], Lori A. Birder[4], Claire H. Mitchell[5] and William C. de Groat[2]

[1] *Department of Anaesthesiology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261, USA*
[2] *Department of Pharmacology and Chemical Biology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261, USA*
[3] *Department of Urology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261, USA*
[4] *Department of Medicine, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261, USA*
[5] *Department of Anatomy and Cell Biology, University of Pennsylvania School of Dental Medicine, Philadelphia, PA 19104, USA*

Key points

- ATP is released through pannexin channels into the lumen of the rat urinary bladder in response to distension or stimulation with bacterial endotoxins.
- Luminal ATP plays a physiological role in the control of micturition because intravesical perfusion of apyrase or the ecto-ATPase inhibitor ARL67156 altered reflex bladder activity in the anaesthetized rat.
- The release of ATP from the apical and basolateral surfaces of the urothelium appears to be mediated by separate mechanisms because intravesical administration of the pannexin channel antagonist Brilliant Blue FCF increased bladder capacity, whereas I.V. administration did not.
- Intravesical instillation of small interfering RNA-containing liposomes decreased pannexin 1 expression in the rat urothelium *in vivo* and increased bladder capacity.
- These data indicate a role for pannexin-mediated luminal ATP release in both the physiological and pathophysiological control of micturition and suggest that urothelial pannexin may be a viable target for the treatment of overactive bladder disorders.

Abstract ATP is released from the bladder epithelium, also termed the urothelium, in response to mechanical or chemical stimuli. Although numerous studies have described the contribution of this release to the development of various bladder disorders, little information exists regarding the mechanisms of release. In the present study, we examined the role of pannexin channels in mechanically-induced ATP release from the urothelium. PCR confirmed the presence of pannexin 1 and 2 mRNA in rat urothelial tissue, whereas immunofluorescence experiments localized pannexin 1 to all three layers of the urothelium. During continuous bladder cystometry in anaesthetized rats, inhibition of pannexin 1 channels using carbenoxolone (CBX) or Brilliant Blue FCF (BB-FCF) (1–100 μM, intravesically), or by using intravesical small interfering RNA, increased the interval between voiding contractions. Intravenous administration of BB-FCF (1–100 μg kg^{-1}) did not alter bladder activity. CBX or BB-FCF (100 μM intravesically) also decreased basal ATP concentrations in the perfusate from non-distended bladders and inhibited increases in ATP concentrations in response to bladder distension (15 and 30 cmH_2O pressure). Intravesical perfusion of the ATP diphosphohydrolase apyrase (2 U ml^{-1}), or the ATPase inhibitor ARL67156 (10 μM) increased or decreased reflex bladder activity, respectively. Intravesical instillation of bacterial lipopolysaccharides (LPS) (*Escherichia coli* 055:B5, 100 μg ml^{-1}) increased ATP concentrations in the bladder perfusate, and also increased voiding frequency; these effects were suppressed by BB-FCF. These data indicate that pannexin channels contribute to

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distension- or LPS-evoked ATP release into the lumen of the bladder and that luminal release can modulate voiding function.

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Corresponding author J. M. Beckel: Department of Anaesthesiology, University of Pittsburgh School of Medicine, W1353 Thomas E. Starzl Biomedical Science Tower, 200 Lothrop Street, Pittsburgh, PA 15261, USA. Email: jmbeckel@pitt.edu

Abbreviations 18α-GA, 18α-glycyrrhetinic acid; BB-FCF, Brilliant Blue FCF; CBX, carbenoxolone; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; PPADS, pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid; siRNA, small interfering RNA; VNUT, vesicular nucleotide transporter.

Introduction

Purinergic signalling is considered to play a significant role in sensory functions in the urinary bladder (Cockayne *et al.* 2000; Burnstock, 2001; Vlaskovska *et al.* 2001; Apodaca *et al.* 2007; Birder, 2010). For example, ATP is known to be released from the urothelium in response to mechanical (distension) (Ferguson *et al.* 1997; Lewis & Lewis, 2006; Mochizuki *et al.* 2009; Miyamoto *et al.* 2014), as well as chemical stimuli (capsaicin, acetylcholine, bradykinin) (Birder *et al.* 2001; Birder *et al.* 2002; Birder *et al.* 2003; Chopra *et al.* 2005; Girard *et al.* 2008; Kullmann *et al.* 2008; Beckel & Birder, 2012; Mansfield & Hughes, 2014; Sui *et al.* 2014). It has been hypothesized that ATP modulates the sensations of fullness felt in the bladder through depolarizing actions on afferent nerve terminals adjacent to the urothelium (Rong *et al.* 2002; Birder & Andersson, 2013). Although it is uncertain what influence this ATP release may have on normal bladder function, purinergic signalling has been shown to play a significant role in the emergence of bladder pathology (Cockayne *et al.* 2000; Yu & de Groat, 2008). For example, ATP release from urothelial cells is significantly elevated after stimulation by *Escherichia coli* bacteria, mimicking a urinary tract infection (Säve & Persson, 2010). ATP is also elevated in the urine of patients suffering from overactive bladder (Silva-Ramos *et al.* 2013) or interstitial cystitis/painful bladder syndrome (Sun & Chai, 2006), disorders characterized by increased bladder sensations, an increased frequency of voiding, and pain, in the case of interstitial cystitis/painful bladder syndrome. Intravesical administration of ATP in animals also causes increased frequency of voiding (Pandita & Andersson, 2002; Nishiguchi *et al.* 2005). Given this apparent role for purinergic signalling in the pathology of the urinary bladder, insights into the mechanism of ATP release from the urothelium represent a critical first step in developing potential treatments for these disorders.

Established dogma regarding the mechanism of ATP release from cells or neurons involves packaging the transmitter into vesicles via a transporter and release by fusion of vesicles with the plasma membrane in response to various stimuli (Burnstock & Verkhratsky, 2012). Evidence for a role of exocytosis in the urothelial release of ATP includes a dependence on extracellular calcium (Birder *et al.* 2003) and inhibition of release by incubation with blockers of vesicular trafficking and fusion, such as brefeldin A (Sui *et al.* 2014) and onabotulinum toxin A (Smith *et al.* 2005). Additionally, staining of urothelial cells with quinacrine or the fluorescent analogue of ATP, Mant-ATP, reveals punctate labelling, which is presumed to reflect the presence of ATP-laden vesicles (Wang *et al.* 2005; Nakagomi *et al.* 2011). However, there is some debate regarding the interpretation of these results because quinacrine and Mant-ATP have been shown to label lysosomes and other acidic vesicles not related to transmitter secretion (Miller *et al.* 1999; Dou *et al.* 2012). Additionally, blocking vesicular fusion with the plasma membrane by brefeldin A or onabotulinum toxin A would also prevent the trafficking to the plasma membrane of other transporters or channels important for ATP release. Recently, a number of non-vesicular mechanisms of ATP release have been identified in various cell types, including voltage-dependent anion channels, maxi-anion channels, connexin hemichannels, bestrophins, the cystic fibrosis transmembrane conductance regulator and the CALHM1 channel (Burnstock & Verkhratsky, 2012; Taruno *et al.* 2013). These mechanisms are also attracting attention as important contributors to purinergic signalling in a number of cell types.

A non-vesicular mechanism of ATP release mediated by pannexin channels has been identified in recent years as a key contributor to purinergic signalling in various cells. The pannexin protein is functionally related to the family of connexin proteins that make up gap junctions in mammalian cells; however it shares very little homology with them (Barbe *et al.* 2006). Pannexin channels, unlike connexin channels, do not join together to form gap junction pores between neighbouring cells but, instead, form a pore directly between the cytosol and the extracellular space. This pore has a large conductance and is of sufficient size when open to allow the passage of molecules such as ATP or the fluorescent dye, propidium iodide, which are too large for passage through classic ion channels (Bao *et al.* 2004). Pannexin channels have been implicated in the release of ATP from gustatory receptor

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cells (Huang *et al.* 2007), retinal ganglion cells (Xia *et al.* 2012), astrocytes (Beckel *et al.* 2014), bronchial epithelial cells (Ransford *et al.* 2009), erythrocytes (Locovei *et al.* 2006), T cells (Schenk *et al.* 2008), hippocampal neurons (Zoidl *et al.* 2007) and renal tubule epithelial cells (Hanner *et al.* 2012). Given its emergence as a common mechanism for ATP release throughout the body, we aimed to determine whether ATP release from the urothelium is also mediated through pannexin channels.

One condition in particular where pannexin-mediated ATP release may play an important role is inflammation. Purinergic signalling has been shown to be important for the induction of the inflammatory response and hyperalgesic behaviour exhibited by rats after injection of formalin in the paw (Cockayne *et al.* 2000). Pannexin channels may be an important upstream effector of these responses because ATP release through pannexin channels, and subsequent activation of purinergic receptors, represents an important step in the activation of the inflammasome and release of pro-inflammatory cytokines (Pelegrin & Surprenant, 2006). Given that both ATP and cytokines have been implicated in the hyperexcitability of bladder afferent nerves following inflammation (Fu & Longhurst, 1999; Dang *et al.* 2008; Yu & de Groat, 2008; Stemkowski, 2012), it is possible that pannexin channels are important for the development of bladder hyperactivity during a lower urinary tract infection. Therefore, the present study also investigated whether ATP released from the urothelium in response to bacterial toxins is mediated by pannexin channels. The identification of a role for pannexins in bladder pathophysiology could lead to the development of new treatments for bladder disorders that involve urinary urgency and pain.

Methods

Animal care and use

A total of 45 female Sprague–Dawley Rats (Harlan Laboratories, Frederick, MD, USA) and three female Long–Evans rats, weighing 225–275 g were used in these studies. Animals were housed in the University of Pittsburgh's AAALAC-accredited animal housing facility in a temperature-controlled room under a 12:12 h light/dark cycle with food and water *ad libitum*. For all surgical procedures, animals were anaesthetized using either isoflurane (4% for induction, followed by 1.5–2.0% for maintenance), ketamine (100 mg kg^{-1}, I.P.) or urethane (1.2 g kg^{-1}, S.C.), as described below. For experiments utilizing animal tissue, animals were either anaesthetized using one of the methods described above or killed by CO_2 inhalation before tissue harvesting. All procedures were performed in strict accordance with the National Research Council's 'Guide for the Care and Use of Laboratory Animals' and were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Solutions and reagents

All drugs and reagents were purchased from Sigma-Aldrich (St Louis, MO, USA), with the exception of ARL67156, which was purchased from Tocris, Inc. (Minneapolis, MN, USA). The composition of the Krebs buffer used for ATP release and cystometry experiments is (in mM): 118 NaCl, 4.8 KCl, 1.2 KH_2PO4, 1.2 $MgSO_4$, 11 D-glucose, 25 $NaHCO_3$, 2.5 $CaCl_2$ (pH 7.4) ~300 mOsmol. The anti-pannexin1 antibody (rabbit polyclonal) was obtained from Alomone Labs (Jerusalem, Israel). The cytokeratin-20 antibody (mouse monoclonal) and the fluorescent secondary antibodies were purchased from Abcam (Cambridge, MA, USA).

RNA extraction and RT-PCR

Bladders were removed from isoflurane anaesthetized rats, placed in oxygenated Krebs solution, cut open longitudinally from the urethra to the dome, and pinned flat in a Sylgard-coated dish (Dow Corning, Midland, MI, USA) containing Krebs solution with the urothelium facing up. Using fine scissors and forceps, the urothelium was gently teased away from the underlying smooth muscle and placed into Trizol (Invitrogen, Grand Island, NY, USA), homogenized, and total RNA was extracted in accordance with the manufacturer's instructions. RNA purity and concentration was determined using a UV spectrophotometer. cDNA was created using the High Capacity cDNA Reverse Transcription Kit from Applied Biosystems (now Invitrogen) with 1 μg of total RNA. PCR was performed with a HotStarTaq Master Mix Kit (Qiagen, Valencia, CA, USA) using sequence specific primers for the *Panx1*, *Panx2*, *Panx3* and *Actb* (β-actin) genes, which were designed in-house using previously published sequences (NCBI, Bethesda, MD, USA) and online primer design software (Primer3; http://biotools.umassmed.edu/bioapps/primer3_www.cgi). Primers were: *Panx1* (NM_199397.2) L: 5'-GCTGTGGGCCATTATGTCTT-3', R: 5'-GCAGCCAGAGAATGGACTTC-3'; *Panx2* (NM_199409.2) L: 5'-CAAGGGGAGTGGAGGTGATA-3', R: 5'-GTGGGGTATGGGATTTCCTT-3'; *Panx3* (NM_199398.1) L: 5'-TTTCCCTTGCTAGAGCGGTA-3', R: 5'-GGGGCTCTAGAAGGCTCTGT-3'; *Actb* (NM_031144.3) L: 5'-AGCCATGTACGTAGCCATCC-3', R: 5'-ACCCTCATAGATGGGCACAG-3'. PCR was performed using a PTC-100 Thermal Cycler (MJ Research; Bio-Rad, Hercules, CA, USA) with the profile: 95°C for 10 min; 40 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min; and 72°C for 10 min. PCR

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products were visualized on a 2.0% agarose gel in TBE buffer using ethidium bromide as a nucleic acid stain. Expected product sizes were: *Panx1*: 234 bp; *Panx2*: 264 bp; *Panx3*: 104 bp; *Actb*: 115 bp. Sequences of positive PCR products were confirmed by the University of Pittsburgh DNA Sequencing Core facility and by comparison with the published sequence using BLAST (http://blast.ncbi.nlm.nih.gov).

Immunofluorescence staining

Rat bladders were removed from isoflurane anaesthetized animals and pinned in Sylgard dishes as described above. After washing with Krebs solution, the bladders were immersed in 0.2 M sodium cacodylate buffer containing 4% (w/v) paraformaldehyde for 15 min at room temperature. Bladders were then washed in phosphate-buffered saline (PBS) 3 times and placed in 30% sucrose in PBS overnight at 4°C for cyroprotection. The next morning, the tissue was cut into quarters and quickly frozen in standard cryomolds containing OCT and stored at −80°C until sectioning. The frozen tissue was sectioned at 10 μm using a cryostat and placed onto charged glass slides. After washing 3 times with PBS, the tissue was permeabilized by incubations with 0.1% Triton X-100 and 0.05% SDS and washed 3 times with PBS again. After blocking for 2 h at room temperature with a PBS solution containing 10% normal goat serum, 0.7% (w/v) fish skin gelatin and 0.025% saponin, the tissue was incubated overnight at 4°C in a humidity chamber with primary antibodies against pannexin 1 and cytokeratin-20, diluted in blocking buffer. The concentrations of antibodies were 1:250 each. The next morning, the tissue was washed with PBS and blocking solution, incubated in the secondary antibodies (goat anti-rabbit FITC and goat anti-mouse rhodamine, 1:750 dilution in blocking solution each) for 2 h at room temperature and washed again in PBS and blocking solution. Cell nuclei were stained by incubating for 1 min with a 1:10 000 dilution of 4',6-diamidino-2-phenylindole in PBS. Following another round of washing with PBS, the tissue was post-fixed for 15 min with 4% paraformaldehyde in 0.2 M sodium cacodylate buffer and then coverslipped using the SlowFade Gold mounting medium (Invitrogen). Staining was visualized using a DM5000B fluorescence upright microscope (Leica Microsystems, Wetzlar, Germany) and images were captured using the Leica Application Suite software, version 4.2. Multicolored images were created using Photoshop CS4 (Adobe Systems, San Jose, CA, USA) by adding separate micrographs of each fluorophore and adding them to the appropriate RGB channel of a new file. Additionally, each micrograph was adjusted for brightness and contrast so that the published image most closely represented what was observed directly under the microscope. Antibody specificity was confirmed in a separate experiment by preadsorbing the antibody with the control antigen (10-fold greater concentration) overnight before using in the staining procedure outlined above. No staining above background was observed when the preadsorbed antibody was used (results not shown).

Bladder cystometry

In urethane (1.2 g kg^{-1}, S.C.) anaesthetized rats, the urinary bladder was exposed via a mid-line incision and a catheter (intramedic tubing, PE-60) was inserted into the bladder lumen through a small incision in the bladder dome and secured with a purse-string suture. The catheter was connected via a three-way stopcock to a syringe pump for fluid infusion and to a pressure transducer connected to a computerized data acquisition system (DataQ Inc., Akron, OH, USA) to record changes in bladder pressure. The ureters were also tied and cut proximal to the ligation to prevent urine from the kidneys filling the bladder. A moistened wick of sterile gauze was inserted into the mid-line incision and sewn in place to drain urine from the abdominal cavity. In some experiments drugs were administered via a catheter (PE-10) inserted into the internal jugular vein. Krebs solution was infused intravesically (0.08 ml min^{-1}) for a control period (generally 1–2 h) to establish baseline bladder activity. After the control period, the bladder infusate was switched to a Krebs solution containing the compound of interest and measurements were taken for an additional 1 h. For I.V. administration, each drug, dissolved in isotonic saline, was given in a concentrated bolus (0.1 ml) followed by a bolus of isotonic saline to clear the line (0.1 ml) and cystometric readings were taken for 1 h.

In vivo ATP measurement

Rats anaesthetized with urethane (1.2 g kg^{-1}, S.C.) were prepared as described above for cystometry and an additional 18 gauge angiocatheter (without needle) was inserted into the bladder through the external urethral orifice to allow for fluid collection. Krebs solution was infused through the catheter in the bladder dome at 0.2 ml min^{-1} and allowed to drain through the urethral catheter for a period of 1 h to wash out any ATP released by the catheter implantation. Samples of the perfusate (100 μl) were then taken from the urethral catheter for measurement of ATP concentrations using a luminometer and the luciferin-luciferase assay (Sigma-Aldrich). To determine luminal ATP concentrations during bladder distension, a luerlock plug was screwed into the transurethral catheter to block outflow of perfusate and allow the bladder

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to fill. To prevent reflex bladder contractions during filling, each animal was treated with hexamethonium bromide (50 mg kg^{-1}), a ganglionic blocking agent, I.P. during the control washout period. Bladder pressure was measured using a pressure transducer connected to a computer via a data acquisition system. When the bladder reached the desired pressure, the plug was removed, the perfusate collected and samples were measured for ATP concentration. To examine the effects of various compounds on luminal ATP concentrations, the Krebs perfusate was replaced by the appropriate drug solution (dissolved in Krebs). Luminescence readings were converted to ATP concentrations using a standard curve consisting of samples of known ATP concentrations. Interference with the luciferin-luciferase assay by the drugs utilized in the study [ARL67156, apyrase, Brilliant Blue or lipopolysaccharide (LPS)] was corrected by performing standard curves in solutions containing those substances. To account for variability between animals, the ATP concentrations were all normalized to the average of the readings taken with Krebs perfusion without bladder distension (0 cmH_2O pressure) in each animal.

Knockdown of *Panx1 in vivo* using small interfering RNA (siRNA) in liposomes

To knockdown pannexin 1 expression in the urothelium of rats, we utilized a trio of siRNA duplexes (TriSilencer-27) available from OriGene (Rockville, MD, USA). TriSilencer-27 consists of three siRNA duplexes of 27 bp each that are processed by the DICER protein in mammalian cells to 21 base siRNA strands specifically designed to bind the *Panx1* mRNA and cause degradation through the RISC complex (Tijsterman & Plasterk, 2004). Each duplex was reconstituted in the provided buffer in accordance with the manufacturer's instructions to a concentration of 20 μM and then the duplexes were diluted in liposomes for use as a transfection agent *in vivo*, as described previously (Kashyap *et al.* 2013). Briefly, cationic liposomes composed of DOTAP were made by the thin film hydration method, in which lipid film was hydrated with nuclease-free water to a lipid concentration of 3 mM, aliquoted in separate vials and stored at −20°C prior to the day of use. On the day of experimentation, a frozen liposome aliquot was thawed at room temperature for 1 h and diluted 10-fold with RNase-free PBS (containing Ca^{2+} and Mg^{2+}). Concentrated siRNA duplexes or scrambled siRNA controls were diluted to a final concentration of 200 nM each (600 nM of siRNA total) and mixed with prepared liposomes, and the resulting mixture was incubated for 1 h at room temperature to allow the duplexes to complex with the liposomes. When this incubation was complete, the rats were anaesthetized with isoflurane and 18 gauge angiocatheters (without needle) were inserted into the bladder through the urethral orifice. Next, 0.5 ml of the siRNA-liposome complex was infused into the bladder through the urethral catheter with a 1 ml syringe and allowed to incubate for 1 h. The catheter was then removed and the animals were allowed to recover from anaesthesia before returning them to the animal facility. This procedure was repeated 2 days after the initial treatment. On day 5, the animals were anaesthetized with urethane and cystometry was performed as described above. Following cystometry, the animals were killed, and the bladders were removed and processed for pannexin 1 expression using western blotting.

For western blotting, protein was extracted from homogenized bladder tissue using Trizol (Invitrogen) in accordance with the manufacturer's instructions and resuspended in RIPA buffer containing 2% SDS and complete protease inhibitor cocktail. After centrifugation (13 000 r.p.m. 10,000$\times g$ for 15 min at 4ºC), protein concentrations of each sample were determined using the Pierce BCA protein assay (Thermo Scientific, Rockford, IL, USA). After denaturation (100ºC for 5 min) in the presence of Laemmli sample buffer, lysate from each sample was separated on a 4–15% TGX Stain-Free SDS-PAGE gel (Bio-Rad). As a loading control, total protein per sample was determined using Bio-Rad Stain Free SDS-PAGE gel technology. UV-activated protein fluorescence was imaged on a ChemiDoc MP (Bio-Rad). After proteins were transferred to polyvinylidene fluoride membranes, the membranes were incubated in 5% (w/v) dried milk dissolved in TBS-T (20 mM Trizma, 137 mM NaCl, 0.1% Tween-20, pH 7.6), rinsed with TBS-T, and incubated overnight at 4ºC with primary antibody [rabbit polyclonal (AB124131); Abcam] diluted in TBS-T containing 5% (w/v) milk. After washing in TBS-T, the membranes were incubated with secondary antibody (donkey anti-rabbit HRP; Southern Biotech, Birmingham, AL, USA) for 1 h in 5% (w/v) Milk TBS-T, washed, and incubated in WesternBright Quantum (Advansta, Menlo Park, CA, USA) and then imaged on a ChemiDoc MP (Bio-Rad). A single immunoreactive band was observed for pannexin 1 at 52 kDa (expected size 45–48 kDa). The volume (intensity) of each protein species was determined and normalized to total protein using Image Lab software (Bio-Rad).

To determine the distribution of intravesically instilled siRNA, animals were treated with a red fluorescent siRNA (400 nM) in liposomes, as described above. These animals (n = 2) were killed by cardiac transfusion with paraformaldehyde after 24 h. Their bladders were removed, frozen in OCT, sectioned using a cryostat, and mounted on glass slides to visualize the distribution of fluorescent signal in the bladder by microscopy.

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Statistical analysis

Data are reported as the mean ± SEM. Statistical analysis used either a Student's *t* test or one-way ANOVA with the appropriate *post hoc* test. ATP measurements, which included data from various intravesical pressures with and without intravesical drugs, were analysed using a two-way ANOVA with the appropriate *post hoc* test. Although the data are expressed as the percentage change from the control, all statistical analyses were performed on raw data. $P < 0.05$ was considered statistically significant. Data were compiled and statistically analysed using Prism 6 software (GraphPad, Inc., La Jolla, CA, USA).

Results

Pannexin channel expression in the rat urothelium

To examine pannexin channel expression in the urothelium, PCR was performed on RNA extracted from urothelial tissue that was surgically removed from the rat bladder. Standard qualitative PCR using primers designed against the three known subtypes of pannexin channel (*Panx1*, *Panx2* and *Panx3*) revealed positive products for pannexin 1 and 2, but not for pannexin 3 in Sprague–Dawley rats (Fig. 1*A*, left). PCR on urothelial tissue taken from Long–Evans rats revealed positive products for pannexin 1 and 3, but not for pannexin 2 (Fig. 1*A*, right), suggesting that strain differences in pannexin channel expression exist. Immunofluoresence staining for pannexin 1 channels in bladder tissue of Sprague–Dawley rats revealed strong staining in cells adjacent to the lumen of the bladder (Fig. 1*B*). Moreover, co-localization studies with antibodies against cytokeratin-20, which label the umbrella cells of the urothelium, revealed that pannexin 1 channels are expressed both in the umbrella cell layer and in the cell layers immediately below the cytokeratin-20 staining (Fig. 1*B*, merged image). This indicates that pannexin 1 is expressed throughout all three layers of the urothelium of the rat (umbrella, intermediate and basal).

Suppression of reflex bladder activity during intravesical perfusion, but not after systemic administration, of pannexin channel antagonists

ATP, instilled intravesically, can excite bladder reflexes in the rat (Pandita & Andersson, 2002; Nishiguchi *et al.* 2005). To determine whether endogenous ATP, released luminally through pannexin channels, plays a role in modulating bladder activity, we performed cystometry in anaesthetized rats. As shown in Fig. 2, intravesical instillation of the pannexin channel antagonists BB-FCF (BB-FCF, 1–100 μM) or carbenoxolone (CBX, 1–100 μM) suppressed reflex bladder activity, lengthening the time between voiding bladder contractions (by up to 155% of control for BB-FCF, 160% for CBX; $n = 6$ and 5 rats, respectively). This suppression was concentration-dependent, began within one or two contractions after infusion began, and was sustainable throughout the length of perfusion (at least 1 h). The inhibitory effects of BB-FCF could be washed out by returning the perfusate to Krebs solution, again occurring within one or two contractions after the washout began.

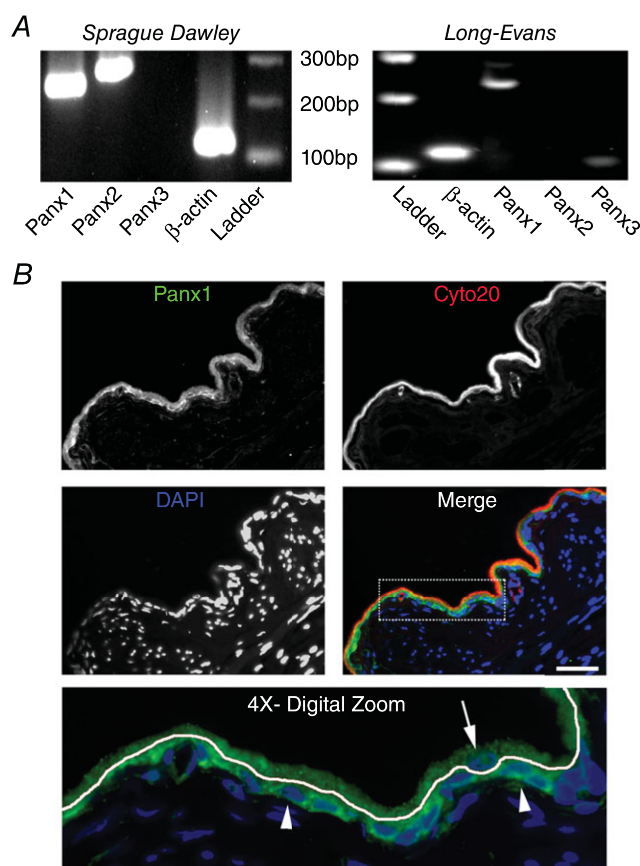

Figure 1. The rat urothelium expresses pannexin 1 channels
A, products of PCR amplification of pannexin genes in urothelial tissue of Sprague–Dawley (SD, left) and Long–Evans (LE, right) rats. Note the positive PCR products for *Panx1* and *Panx2* for SD and *Panx1* and *Panx3* for LE. Positive products for the housekeeping gene β-actin are also shown as a positive control. Expected product sizes are listed in the Methods. Results shown are typical of those obtained from three separate rats. *B*, immunofluorescence localization of pannexin 1 in whole bladder tissue sections of a SD rat. Pannexin 1 (*Panx1*) staining is shown upper left, whereas the umbrella cell marker cytokeratin-20 (Cyto 20) is shown upper right. The nuclear stain 4′,6-diamidino-2-phenylindole is shown middle left. A colourized, merged imaged is shown middle right. Scale bar = 50 μm. Bottom: a 4× digital zoom of the area denoted by a dashed rectangle (middle right). The white line denotes the boundary of the umbrella cells as determined by cytokeratin staining. Note that pannexin 1 is expressed in the umbrella cell layer (white arrow), as well as the underlying intermediate and basal cell layers (arrowheads). Results shown are typical of those obtained from three separate rats.

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Intravesical administration of BB-FCF also significantly increased the pressure threshold required to activate a micturition contraction (up to 138% of control at 100 μM, $P < 0.05$) (Fig. 2*E*). Peak micturition pressure was decreased slightly at the highest concentration of BB-FCF (82.9% of control, $P < 0.05$); however, this effect could not be washed out following a return of the perfusate to Krebs solution (Fig. 2*F*).

ATP is also considered to be released from the basolateral surface of the umbrella cells, where it can act directly on purinergic receptors on afferent nerves to increase their excitability. To determine whether this basolateral release is mediated through pannexin channels, we administered BB-FCF I.V. in five animals. All three doses (1, 10 and 100 μg kg^{-1}) had no statistically significant effect on reflex bladder activity ($P = 0.97$; $n = 5$) (Fig. 3). By contrast, I.V. administration of the purinergic antagonist pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS; 3 μg kg^{-1}), did increase the intercontraction interval by 35.7% ($P = 0.018$; $n = 4$). Intravesical administration of PPADS also increased the intercontraction interval at concentrations of 0.1–10 μM ($n = 4$ each) (Fig. 3*C*).

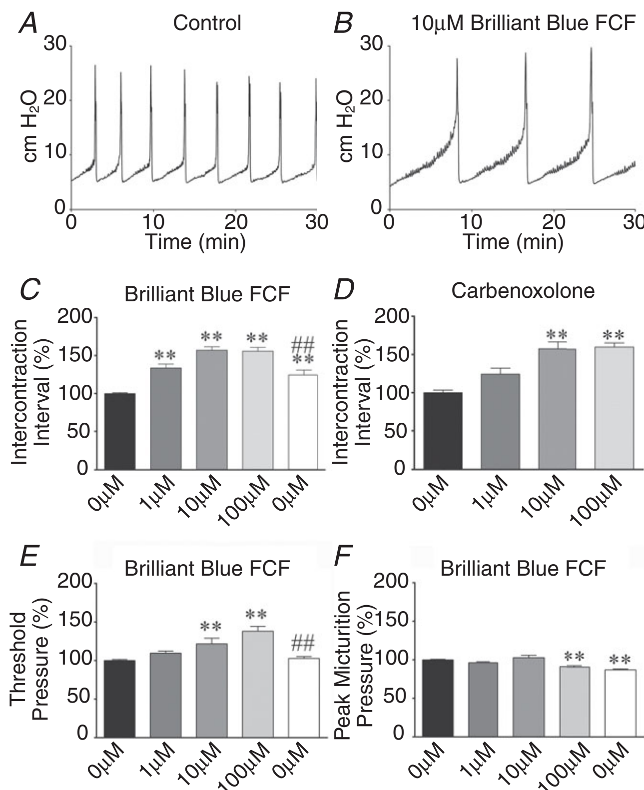

Figure 2. Intravesical perfusion of pannexin channel antagonists suppresses reflex bladder activity in the rat
A and *B*, representative tracings of bladder cystometrograms in the anaesthetized rat taken before and after BB-FCF. *C*, summary of the measured intercontraction intervals during cystometry experiments involving intravesical perfusion of BB-FCF (1–100 μM; $n = 6$ rats) 0 μM = Krebs solution without drug. *D*, summary of the measured intercontraction intervals during cystometry experiments involving intravesical perfusion of carbenoxolone (1–100 μM; $n = 5$ rats). *E*, summary of the measured micturition threshold pressures during cystometry experiments involving intravesical perfusion of BB-FCF ($n = 6$). *F*, summary of the measured peak micturition pressures during cystometry experiments involving intravesical perfusion of BB-FCF ($n = 6$). $^{**}P < 0.05$ compared to control by one-way ANOVA with Tukey's *post hoc* test. $^{\#\#}P < 0.05$ compared to 100 μM BB-FCF.

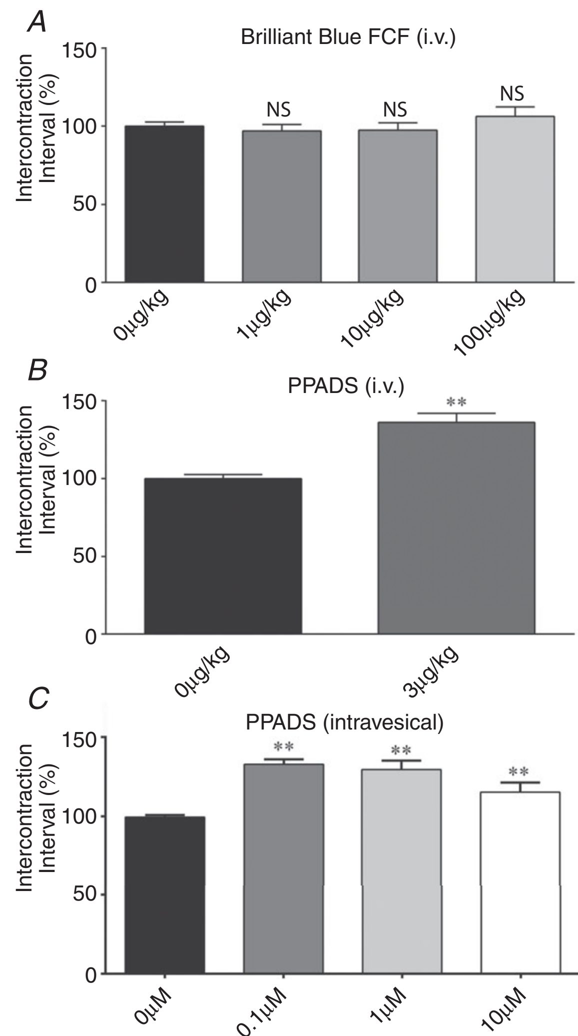

Figure 3. Intravenous administration of pannexin channel antagonists does not suppress bladder reflexes
A, summary of the measured intercontraction intervals during cystometry experiments involving I.V. administration of BB-FCF (1–100 μg kg^{-1}; $n = 5$ rats). 0 μg kg^{-1} = Krebs solution without drug. NS, not significant. *B*, summary of the measured intercontraction intervals during cystometry experiments involving I.V. administration of the purinergic antagonist PPADS (3 μg kg^{-1}; $n = 4$ rats). *C*, summary of the measured intercontraction intervals during cystometry experiments involving intravesical administration of PPADS (0.1–10 μM; $n = 4$ rats). 0 μM = Krebs solution without drug. $^{**}P < 0.05$ compared to control by an unpaired Student's *t* test.

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In vivo measurement of ATP release into the rat bladder lumen in response to stretch

ATP concentrations in the lumen of the bladder at various distension pressures were measured using the luciferin-luciferase assay. In the absence of bladder distension, when the bladder was constantly perfused with Krebs solution and the transurethral catheter was left open to collect the perfusate, ATP was detected at a basal level (24.5 ± 3.2 nM; $n = 13$ rats). Distension of the bladder to a pressure of 15 or 30 cmH_2O, accomplished by closing the transurethral catheter during filling, increased ATP concentrations in the perfusate by 1.8- and 2.4-fold, respectively (n = 5 and 9 rats) (Fig. 4). To determine whether this ATP release was mediated through pannexin channels, we again utilized the two pannexin channel antagonists, CBX or BB-FCF. Because both antagonists demonstrated maximal inhibition of bladder reflexes in the experiments above at a concentration of 100 μM, we used that concentration in an attempt to block ATP release. Both antagonists decreased basal and distension-induced ATP concentrations in the perfusate. Basal concentrations of ATP, in the presence of BB-FCF or CBX, were decreased by 40–50% compared to the control. ATP concentrations in response to distension were also diminished by 55–60% compared to the control ($n = 5$ and 3 rats for BB-FCF and CBX, respectively).

Because pannexin channel antagonists can have non-specific effects, such as inhibition of gap junctions formed by connexin 43, we also tested the ability of the gap junction blocker 18α-glycyrrhetinic acid (18α-GA) to decrease ATP concentrations in the bladder perfusate. As shown in Fig. 4, basal and distension-evoked ATP concentrations were not appreciably different from control concentrations in the presence of 50 μM 18 α-GA, which is a concentration that is generally accepted (Chaytor *et al.* 1999) to block connexin 43 channels ($n = 4$).

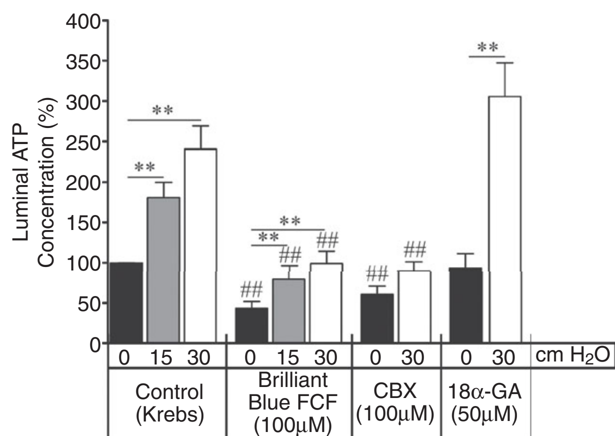

Figure 4. Luminal ATP release in response to stretch is suppressed by pannexin, but not by a connexin channel blocker
Effects of BB-FCF (100 μM; $n = 5$ rats), CBX (100 μM; $n = 3$ rats) and 18 α-GA (50 μM; $n = 4$ rats) on distension-evoked (0–30 cmH_2O) ATP concentrations measured in the urinary bladder perfusate. All concentrations are presented as the percentage change from non-distended (0 cmH_2O) Krebs-infused controls ($n = 13$). **$P < 0.05$ by one-way ANOVA with Tukey's *post hoc* test; ##$P < 0.05$ compared to pressure matched control (Krebs) by an unpaired Student's t test.

Intravesical administration of agents that influence the catabolism of endogenous ATP modulate bladder reflexes in the anaesthetized rat

To further support the hypothesis that ATP released into the lumen of the bladder can modulate bladder reflexes, we intravesically perfused agents that can influence the catabolism of ATP in an attempt to modulate intra-luminal ATP concentrations. Intravesical administration of the ATP diphosphohydrolase, apyrase (2 U ml^{-1}; n = 3), decreased basal ATP concentrations in the bladder perfusate (Fig. 5*A*). Apyrase also eliminated the distension-induced increase in ATP concentration because measurements of ATP concentrations at a pressure of 30 cmH_2O were not statistically different from non-stretched concentrations. Conversely, measurements taken from a separate set of animals demonstrated that the ecto-ATPase inhibitor ARL67156 (10 μM; n = 3) significantly increased ATP concentrations at all bladder pressures. During cystometry in the anaesthetized rat, intravesical administration of apyrase (2 U ml^{-1}) increased the intercontraction interval by 35% ($P < 0.05$; $n = 4$), whereas ARL67156 (10 μM) decreased it by 27% ($P < 0.05$; $n = 4$) (Fig. 5*B* and *C*).

Induction of inflammation by intravesical instillation of bacterial lipopolysaccharides releases ATP through pannexin channels

Intravesical administration of bacterial LPS can cause bladder inflammation through activation of the Toll-like receptor 4 (Song *et al.* 2007). Release of pro-inflammatory cytokines and increases in reflex bladder activity have also been shown to be driven by the release of ATP and activation of purinergic receptors (Säve & Persson, 2010). To determine whether LPS-induced ATP release is mediated through pannexin channels, we examined luminal ATP release *in vivo* during infusion of LPS (100 μg ml^{-1}). LPS increased basal ATP release by 2.2-fold and stretch-mediated release (15 cmH_2O) by 5.3-fold (Fig. 6*A*). Concurrent infusion of BB-FCF (100 μM) significantly diminished, but did not abolish, the LPS-induced increase in ATP concentrations under basal and distended conditions (1.5- and 3.1-fold increases over

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pressure matched controls, respectively). During bladder cystometry, LPS perfusion (100 μg ml^{-1}) significantly excited bladder reflexes (decrease in intercontraction interval of 29.5%, $P < 0.001$; $n = 5$), which was completely reversed during concurrent perfusion with BB-FCF (100 μM, Fig. 6*B*).

Knockdown of pannexin 1 expression in the urothelium using intravesical instillation of liposomes loaded with anti-*Panx1* siRNA increases intercontraction interval and eliminates inhibition of reflex bladder activity by BB-FCF

Because many pannexin channel antagonists can have non-specific actions, we attempted to use siRNA to knockdown pannexin 1 expression as a means of confirming our pharmacological results. We utilized a liposome-based delivery system to instill siRNA intravesically with the goal of transfecting urothelial cells. Liposomes loaded with three different duplexes of siRNA against *Panx1* mRNA were instilled into the bladder of anaesthetized rats for 1 h twice (on days 1 and 3) and cystometry was performed on day 5. Intercontraction intervals of rats treated with the siRNA were 40% longer than those in untreated animals ($P < 0.05$; $n = 3$) (Fig. 7*A*). Bladder activity in animals treated with scrambled siRNA was not significantly different from that of untreated animals ($P = 0.18$; $n = 3$). Additionally, intravesical administration of BB-FCF had no effect on reflex bladder activity in the siRNA treated animals. Western blot analysis of extracted whole bladder protein confirmed that siRNA treatment knocked down pannexin 1 expression by 93% ($P < 0.05$), whereas scrambled siRNA did not significantly alter pannexin 1 expression ($P = 0.156$) (Fig. 7*B*). Micrographs of bladder tissue taken from animals instilled intravesically with fluorescent siRNA suggest that this knockdown is limited to the urothelium because a fluorescent signal is only observed in the urothelial layer (Fig. 7*C*).

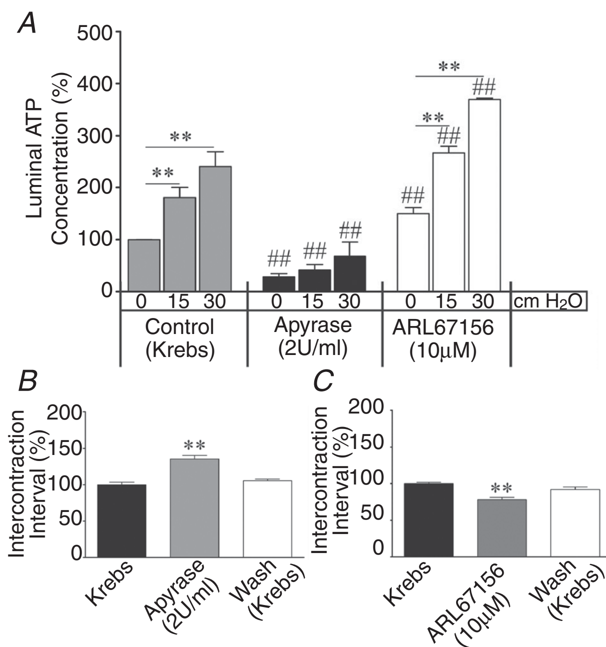

Figure 5. Modulators of ATP catabolism influence luminal ATP concentrations and bladder activity in response to distension
A, effects of apyrase (2 U ml^{-1}; $n = 3$ rats) or ARL67156 (10 μM; $n = 3$ rats) on distension-evoked ATP concentrations in the urinary bladder perfusate. $^{**}P < 0.05$ as measured by one-way ANOVA with Tukey's *post hoc* test. $^{\#\#}P < 0.05$ compared to pressure matched control (Krebs) by an unpaired Student's *t* test. *B* and *C*, effects of intravesical apyrase (*B*) (2 U ml^{-1}; $n = 4$ rats) or ARL67156 (*C*) (10 μM, $n = 4$ rats) on the intercontraction interval during cystometry in the anaesthetized rat. $^{**}P < 0.05$ compared to pressure matched control (Krebs) by an unpaired Student's *t* test.

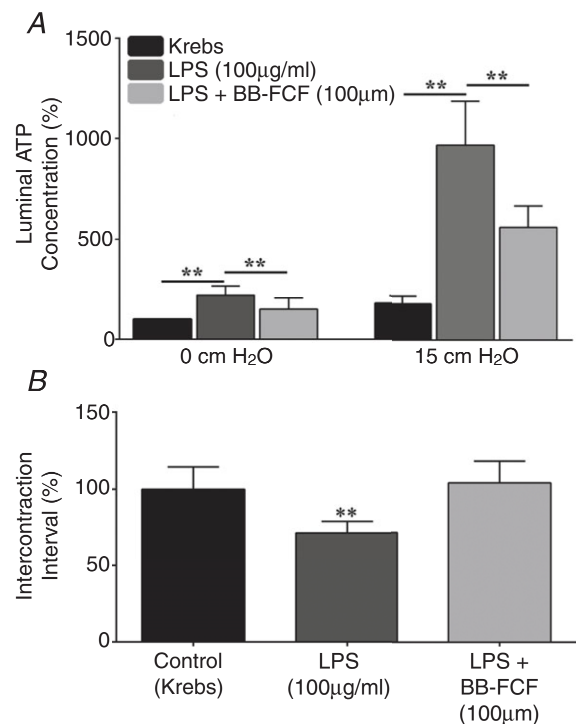

Figure 6. Release of ATP and excitation of reflex bladder activity in the LPS-treated anaesthetized rat is diminished by BB-FCF
A, inhibitory effect of BB-FCF (100 μM; $n = 5$ rats) on ATP concentrations in the urinary bladder perfusate following stimulation with 100 μg ml^{-1} LPS at two different distension pressures (0 and 15 cmH_2O). $^{**}P < 0.05$ compared by two-way ANOVA with Dunnett's multiple comparisons test. *B*, excitatory effect of LPS (100 μg ml^{-1}; $n = 5$ rats) on reflex bladder activity and the ability of BB-FCF (100 μM) to reverse that excitation. $^{**}P < 0.05$ compared by two-way ANOVA with Dunnett's multiple comparisons tests.

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Discussion

ATP, which is an important signalling molecule in the sensory system of the urinary bladder, is released from urothelial cells in response to mechanical and chemical stimuli (Ferguson *et al.* 1997; Birder *et al.* 2001, 2002, 2003; Chopra *et al.* 2005; Lewis & Lewis, 2006; Girard *et al.* 2008; Kullmann *et al.* 2008; Mochizuki *et al.* 2009; Beckel & Birder, 2012; Mansfield & Hughes, 2014; Sui *et al.* 2014) and is considered to directly activate purinergic receptors present on bladder afferent terminals to increase their excitability (Cockayne *et al.* 2000; Vlaskovska *et al.* 2001; Rong *et al.* 2002; Yu & de Groat, 2008). Although numerous studies have identified various stimuli that can release ATP from the urothelium, very little research has been performed to examine the mechanism of this release. Various studies have provided data to suggest a canonical, exocytotic, vesicular mechanism for ATP release, which is blocked following incubation with inhibitors of vesicular transport or fusion, such as brefeldin A or botulinum toxin (Smith *et al.* 2005; Sui *et al.* 2014). However, there also exist, in various cell types, a number of non-vesicular mechanisms of ATP release, including voltage-dependent anion channels, the cystic fibrosis transmembrane conductance regulator channel, the CALHM1 channel and pannexin channels. A review of ATP release mechanisms is provided by Burnstock and Verkhratsky (2012). Pannexin channels are considered to have a significant role in purinergic signalling in many cell types. Pannexin channels form channels between the cytoplasm and the extracellular space, much in the same way as ion channels (Panchin *et al.* 2000; Sosinsky *et al.* 2011). Pannexin channels form very large pores compared to ligand-gated ion channels, having a conductance of ~300 pS, and are permeable to small molecules up to 1 kDa in size, including negatively- and positively-charged dyes, glutamate, and arachidonic acid and its metabolites (Bao *et al.* 2004; Locovei *et al.* 2006; Pelegrin & Surprenant, 2006; Jiang *et al.* 2007; Chekeni *et al.* 2010). The most commonly studied molecules that pass through pannexin channels, however, are the purines, specifically ATP. Pannexin channels mediate ATP release in a growing number of cell types (Locovei *et al.* 2006; Huang *et al.* 2007; Schenk *et al.* 2008; Ransford *et al.* 2009; Hanner *et al.* 2012; Xia *et al.* 2012; Beckel *et al.* 2014). Two recent studies have also implicated pannexin channels in the release of ATP from the urothelium (Negoro *et al.* 2013; Timoteo *et al.* 2014). In the present study, we have shown that ATP, released through pannexin channels in response to distension or inflammation, alters bladder reflexes in the anaesthetized rat.

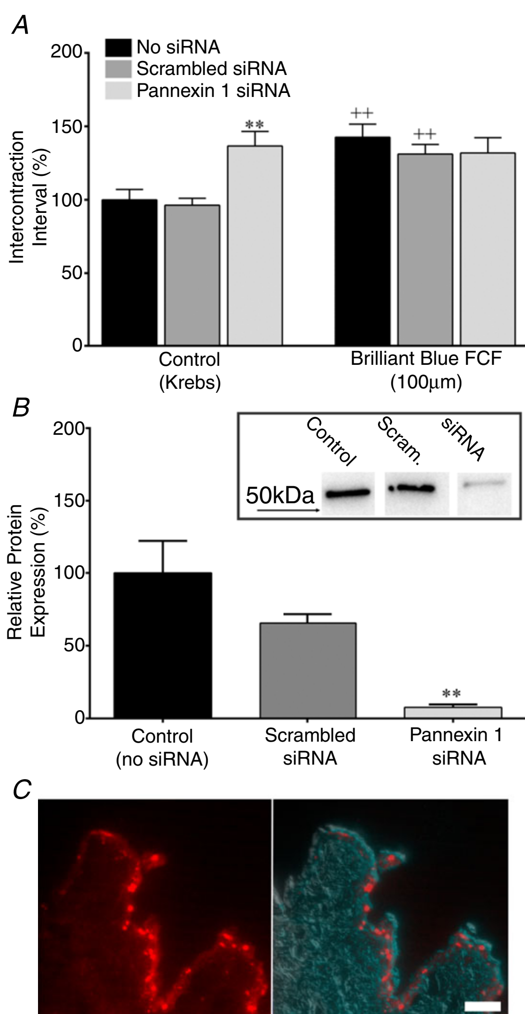

Figure 7. Knockdown of pannexin 1 channels through siRNA suppresses reflex bladder activity and prevents the actions of BB-FF
A, measured intercontraction intervals during cystometry experiments involving BB-FCF (100 μM) in rats treated intravesically for 5 days with siRNA against pannexin 1 or scrambled siRNA as a control ($n = 3$ for each). $^{**}P < 0.05$ compared to Krebs-infused, no siRNA control by two-way ANOVA with Dunnett's multiple comparisons test. $^{++}P < 0.05$ compared to Krebs-infused controls by two-way ANOVA with Dunnett's multiple comparisons tests. *B*, relative pannexin 1 channel expression measured by western blotting in whole rat bladders taken from the cystometrograms (*A*). The inset depicts sample blots. $^{**}P < 0.05$ compared to no siRNA controls by an unpaired Student's *t* test. *C*, fluorescence (left) and differential interference contrast/fluorescence (right) micrographs of urinary bladder tissue from a rat instilled intravesically with liposomes containing fluorescent siRNA (red) as a transfection control. Scale bar = 100 μm.

In the present study, we measured ATP release *in vivo* by perfusing Krebs solution through a catheter in the dome of the bladder and collecting the perfusate through a transurethral catheter. The rationale for using this method was the better examination of ATP release from the urothelium in a physiologically relevant experimental system. Most studies examining ATP release from the urothelium utilize either primary cultured cells or tissue surgically isolated from the underlying detrusor and placed in an Ussing chamber. Both *in vitro* systems have inherent problems;

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cultured cells are generally grown non-polarized and therefore do not truly represent the urothelium *in vivo*. Surgical removal of tissue keeps the urothelium in its transitional, polarized form; however, the act of removal may damage the tissue, initially leading to excess ATP release and, subsequently, to decreased ATP release as the dead cells accumulate. Measuring ATP concentrations *in vivo* allowed us to examine the role of pannexins in distension evoked ATP release in the intact bladder. Examining mechanical stimulation *in vitro* often involves complex stretching devices (e.g. Flexcell; Flexcell International Corp., Burlington, NC, USA) and whether this stretch accurately mimics the strain experienced by the urothelium during bladder distension is a common source of contention. Recording from *in vivo* experiments also allowed us to compare ATP concentrations with the physiological data collected during bladder cystometry. One drawback of measuring ATP concentrations in the lumen of the bladder is that the volume of samples taken at different distension pressures is not constant (i.e. the volume of the sample for a distended bladder is higher than that of an undistended bladder). Therefore, an aliquot taken from the distended sample may be underestimating the amount of ATP as a result of dilution caused by larger volumes. However, we have shown a significant increase in luminal ATP concentrations during distension, and thus we consider this to be of minor concern compared to the drawbacks of *in vitro* recording.

Athough pannexin channels share little homology with connexin channels, they do share a similar pharmacological profile. Therefore, many antagonists against pannexin channels also have effects on connexin gap junctions. For example, carbenoxolone, which we applied at 100 μM as a pannexin channel antagonist, has also been reported to block connexin channels at higher concentrations (Goldberg *et al.* 1996). To avoid non-specific effects, we also used the dye BB-FCF, a compound recently discovered to inhibit pannexin channels without actions on connexin channels (Wang *et al.* 2013). BB-FCF should not be confused with the P2X7 antagonist Brilliant Blue G (BBG), a compound with a very similar structure. Although BBG can have actions on P2X7 and pannexin channels (Qiu & Dahl, 2009), BB-FCF does not have any actions on P2X7 channels (Wang *et al.* 2013). Our data demonstrating that BB-FCF decreased ATP concentrations in the bladder perfusate, whereas the specific antagonist of connexin channels, 18 α-glycyrrhetinic acid, did not, comprises strong evidence indicating that pannexin channels contribute to distension-evoked ATP release into the lumen of the urinary bladder. However, because non-specific pharmacological effects cannot be completely ruled out, we also examined the effect of siRNA-mediated knockdown of pannexin 1 on reflex bladder activity. Knockdown of pannexin 1 inhibited bladder reflexes to the same degree as a maximal concentration of BB-FCF. The addition of the antagonist to siRNA treated animals had no further inhibitory effect, confirming that BB-FCF is working via actions on pannexin channels.

It is interesting to note that the present study indicates a role for luminal ATP in the modulation of micturition reflexes (Fig. 8). This is in contrast to the established hypothesis that ATP released from the basal surface of urothelial cells in response to chemical and mechanical stimuli acts directly on purinergic receptors present on afferent nerve terminals (Cockayne *et al.* 2000; Burnstock, 2001; Apodaca *et al.* 2007; Birder, 2010). Our data indicate that ATP concentrations in the bladder lumen increase with bladder distension and this increase is sensitive to pannexin channel antagonists. Using apyrase and ARL67156, we have confirmed that ATP, and not another transmitter, is responsible for mediating this effect because both of these agents are specific modulators of purine metabolism. Moreover, given the relative size and

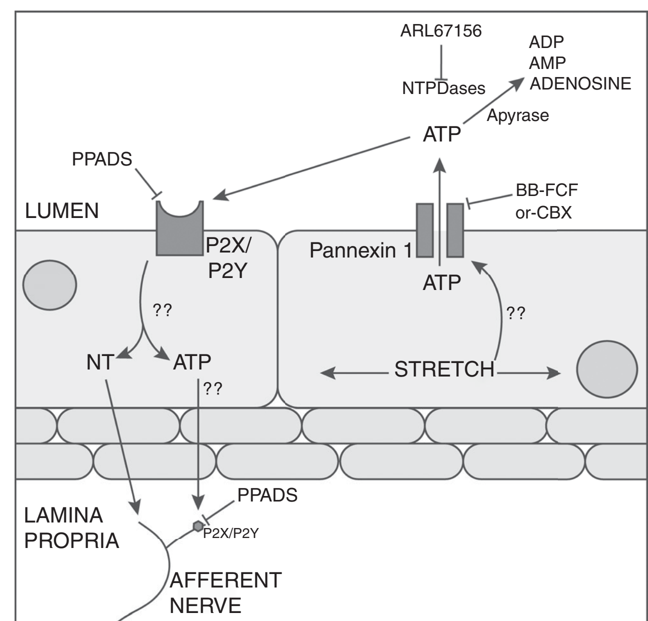

Figure 8. Summary of hypothetical purinergic signalling in the urothelium
Stretch (or stimulation by LPS, not shown) activates pannexin channels, leading to ATP release into the lumen of the bladder by an as yet unknown mechanism. Luminal ATP can be broken down by endogenous ecto-nucleotidases (NTPDases) or, in the present study, exogenous apyrase perfused intravesically. These NTPDases can be inhibited by ARL67156, increasing ATP concentrations. Luminal ATP can act on P2X or P2Y receptors on the urothelium, resulting in the release of neurotransmitters (NT) from the basolateral surface of the urothelium, which act on afferent nerves to increase their excitability. It is possible that ATP is one of these transmitters because systemic administration of the purinergic antagonist PPADS decreases reflex bladder activity. The basolateral release of ATP appears to occur via a mechanism other than pannexin channels because systemic BB-FCF has no effect on bladder reflexes.

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hydrophilic nature of both compounds, it is unlikely that either agent could cross the urothelial barrier to act on sub-urothelial targets.

Bladder conditions such as lower urinary tract infection, interstitial cystitis or an overactive bladder are known to increase ATP concentrations in urine (Sun & Chai, 2006; Säve & Persson, 2010; Silva-Ramos *et al.* 2013). It is thought that this increased luminal ATP plays a role in the aetiology of urgency and pain felt in these conditions. The findings of the present study indicate that this hypothesis may be true because inhibition of luminal ATP release following LPS stimulation normalized the bladder capacity in the anaesthetized rat. More importantly, however, our experiments also indicate a role for luminal ATP in the control of the 'normal', uninfected bladder (Fig. 8). Inhibition or knockdown of pannexin 1 in rats not treated with LPS also suppressed the frequency of bladder reflexes. Most importantly, changing the concentration of endogenous ATP in the lumen using apyrase or ARL67156 also modulated bladder activity in the rat. This suggests that ATP is released into the lumen of the bladder in response to distension, where it acts on purinergic receptors. Presumably, the activation of these receptors would then stimulate the release of a transmitter from the basal surface of the urothelium, which in turn acts on afferent nerves. It should be noted that our research does not rule out ATP as the transmitter that acts on afferents because I.V. administration of PPADS, a non-selective P2X receptor antagonist, inhibited bladder reflexes. It does, however, suggest that any ATP released from the basal surface of the urothelium is not mediated by pannexin channels because I.V. administration of BB-FCF did not affect bladder reflexes. With respect to the purinergic receptors on the urothelium activated by luminal ATP, a number of different purinergic receptors are expressed in urothelial cells (Birder *et al.* 2004; Chopra *et al.* 2008). Supporting our findings, Munoz *et al.* (2011) also demonstrated a role for intravesical ATP in activating bladder sensory pathways. In this case, intravesical administration of ATP increased lumbosacral afferent nerve activity, which was blocked by the systemic administration of the purinergic antagonist suramin. Another more recent study (Timoteo *et al.* 2014) provided evidence for a role for urothelial P2Y6 receptors in the activation of bladder reflexes because intravesical perfusion of the P2Y6 agonist MRS2578 was found to increase voiding frequency. Similar to our data, increased bladder activity following MRS2578 was inhibited by pannexin antagonists administered intravesically but not systemically. This supports our hypothesis suggesting that the release of ATP from the basolateral surface of the urothelium is not mediated by pannexin, and also suggests a positive feedback loop on the apical surface of the umbrella cells via P2Y receptor activation (Fig. 8).

If pannexin channel antagonists, administered systemically, do not block the basolateral release of ATP, then what might be the mechanism for this release? A number of possibilities exist, including vesicular exocytosis. Of particular interest is the recent discovery of the transporter considered to be responsible for loading ATP into secretory vesicles: the vesicular nucleotide transporter (VNUT) (Iwatsuki *et al.* 2009). Very little is known about the role of VNUT in ATP release in urothelial cells; however, the results of a few studies reported in abstracts indicate that VNUT is present in urothelial tissue, as well as cultured urothelial cells, of both rats and humans (Nakagomi *et al.* 2011, 2012). It remains to be seen what role VNUT plays in urothelial ATP release once these initial studies are completed. The presence of multiple mechanisms of ATP release in the urothelium could be the means by which the urothelium releases ATP differentially from the apical or basolateral surfaces of the cell. Additionally, each mechanism may be responsible for ATP release under varying conditions. Multiple mechanisms of ATP release are present in vascular endothelial cells, which release ATP in response to varying stimuli, implying that different mechanisms may be employed in response to different physiological or pathological stimuli (Lohman *et al.* 2012).

In summary, our data indicate that pannexin channels are responsible for the release of ATP into the lumen of the urinary bladder, but not from the basolateral surface of the urothelium. This clarification of differential mechanisms of ATP release from the urothelium will hopefully lead to a greater understanding of how purinergic signalling in the urothelium can play a role in the control of both physiological and pathological bladder activity.

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Additional information

Competing interests

The authors declare that they have no competing interests.

Author contributions

J.B., S.D., P.T., C.M. and W. de G participated in the experimental design. J.B., A.W. and S.D. performed the experiments and collected data. J.B. performed all the data analyses. J.B. wrote the manuscript, with editorial input from S.D., P.T., C.M., A.W., L.B. and W. de G. All authors approved of the final version of the manuscript submitted for publication.

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