Enhanced Purinergic Contractile Responses and P2X1 Receptor Expression in Detrusor Muscle during Cycles of Hypoxia-Glucopenia and Reoxygenation

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1. What is the central question of this study?

Because the bladder in 60 % of patients with detrusor overactivity has increased atropine-resistant contractile responses to nerve stimulation and high intravesical pressure is associated with episodes of hypoxia-glucopenia and reperfusion, we asked whether cycles of hypoxia-glucopenia and reoxygenation increases purinergic signalling in the in vitro rat bladder.

2. What is the main finding and its importance?

Four cycles of hypoxia-glucopenia and reoxygenation augmented atropine-resistant contractile responses of rat detrusor muscle strips subjected to electrical field stimulation via increased purinoceptor P2X1 expression and ATP release. Purinergic antagonists blocked this augmentation, suggesting a possible therapeutic role for purinergic antagonists in the treatment of detrusor overactivity.

ABSTRACT

Bladders from patients with detrusor overactivity have an increased atropine-resistant contractile response to nerve stimulation. The bladder has also been shown to be very susceptible to hypoxia-glucopenia/reperfusion injury leading to the hypothesis that episodes of hypoxia-glucopenia/reoxygenation result in increased atropine-resistant responses to nerve stimulation in the detrusor muscle. Detrusor muscle strips were suspended in a Perspex organ bath chamber of 0.2 ml perfused with Krebs' solution at 37°C aerated with 21 % O_2, 5 % CO_2, balance nitrogen. Hypoxia-glucopenia was induced by switching perfusion to Krebs' solution without glucose, gassed with 95 % nitrogen and 5 % CO_2. Atropine-resistant contractile responses increased by 40.5 ± 7.3 % after four cycles of hypoxia-glucopenia (10 minutes) and reoxygenation (1 hour), whereas α, β-methylene-ATP-resistant responses did not increase. $P2X_1$ receptor expression in the bladder was increased after hypoxia-glucopenia and reoxygenation cycling and ATP release from stimulated

bladder strips during cycling was also increased. Other P2X receptor mediated mechanisms may also be involved in the augmentation of bladder contraction during hypoxia-glucopenia and reoxygenation cycling, as a non-specific P2X antagonist blocked most of the augmented response, whereas a $P2X_1$ specific antagonist prevented only part of the hypoxia-glucopenia and reoxygenation induced augmentation of contractile response.

In conclusion, four cycles of hypoxia-glucopenia and reoxygenation increased the purinergic, but not the cholinergic, contractile responses to nerve stimulation. Increased $P2X_1$ expression and ATP release may have contributed to hypoxia-glucopenia and reoxygenation augmentation of contractile response. Purinergic antagonists may, therefore, be a useful therapeutic option for the treatment of overactive bladder with increased purinergic mediated contractions.

INTRODUCTION

Detrusor overactivity (DO) is diagnosed by urodynamic investigation and is a common cause of urinary incontinence affecting both men and women (Morant *et al* 2008, Wennberg *et al* 2009). It is characterized by spontaneous or provoked contractions of the bladder during the filling phase of the micturition cycle. The International Continence Society also recognizes bladder overactivity (OAB), which is a clinical diagnosis of a symptom complex including urinary frequency, nocturia and urgency (Abrams *et al* 2002). These symptoms are also a manifestation of DO. The prevalence of this condition tends to increase with age (Nordling 2002) and in men, is commonly associated with bladder outlet obstruction (BOO). In women, OAB is often idiopathic but can be associated with distinct neuropathic conditions (Diokno *et al* 1986 , Fultz & Herzog 1996). Detrusor overactivity secondary to outlet obstruction and idiopathic DO result in similar symptoms and it is possible that the cause may be due to a common abnormality.

One possible common cause of bladder dysfunction results from the detection of hypoxia in the bladder wall (Brading & Symes 2004) because the structure and function of the bladder makes it extremely sensitive to hypoxia and subsequent reperfusion (Brading *et al* 1999). In animals, blood flow to the bladder wall has been determined during the normal bladder micturition cycle and during conditions of high intravesical pressure. For example, there appears to be a direct inverse relationship between intravesical pressure and detrusor blood flow, where blood flow decreases as intravesical pressure increases during micturition (Brading & Symes 2004); when the intravesical pressure reached 40 cm of H_2O pressure, detrusor blood flow ceased. Similarly, the human male bladder showed the same effect, where

decreased detrusor blood flow and decreased bladder compliance correlated strongly (Kershen *et al* 2002). Interestingly, these authors noted that blood flow to the human bladder tended to increase with increasing volume and pressure but at maximum pressure, blood flow was significantly decreased.

Recently, the demonstration that elderly patients with lower urinary tract symptoms have decreased perfusion of the bladder neck and prostate (Pinggera *et al* 2008) raised the possibility that reduced blood flow leads to impaired oxygen and/or nutrient exchange in the bladder wall during the normal micturition cycle exacerbated by atherosclerotic changes, may contribute to the development of lower urinary tract symptoms, such as OAB, with advancing age.

In the normal human bladder, contractile responses to nerve stimulation result from the release of acetylcholine from intramural excitatory nerve endings and the activation of muscarinic receptors (Bayliss *et al* 1999). In 60 % of bladder samples from patients with DO either idiopathic or due to outlet obstruction, there was an additional atropine-resistant component to nerve-mediated contractile responses due to activation of purinergic receptors by release of the co-transmitter ATP from the same nerve endings (Wu *et al* 1999). The authors considered addition of this neurotransmitter could change the nature of the bladder contraction and make it more excitable and so ATP could be an important neurotransmitter in relation to bladder disease. This is supported by the observations that the quantity of ATP released from bladder nerves increases with age, along with an increase in the atropine-resistant component of contractile response to nerve stimulation (Yoshida *et al* 2001). These changes may contribute to the increased incidence of OAB with age

(Yoshida *et al* 2001). The cause of the additional purinergic component of nerve-mediated contraction in the human abnormal bladder remains unknown.

The association of OAB resulting from prolonged and repeated hypoxia and reoxygenation injury (Brading *et al* 2004) in conjunction with increased purinergic-mediated detrusor contractions (Bayliss *et al* 1999) led to the hypothesis that it is the repeated cycles of hypoxia and reoxygenation in the bladder wall that results in augmented purinergic neurotransmission, which then contributes to the development of DO and OAB. This study investigates the effect of *in vitro* hypoxia-glucopenia and reoxygenation cycles on the nerve mediated contractile responses in the rat urinary bladder and the effect of purinergic subtype-specific antagonists.

METHODS

Bladder function

Bladders removed from male and female Wistar rats (150g-300g), in accordance with schedule 1 procedure of the Animal (Scientific Procedures) Act 1986, were placed into Krebs' solution (119 mM NaCl, 4.4 mM KCl, 20 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$ and 11 mM glucose (all from Sigma-Aldrich, Poole, Dorset, UK) in distilled water; pH 7.34). Two longitudinal muscle strips per bladder (6 mm x 3 mm x 1 mm) were cut and suspended using fine silk sutures in Perspex organ bath chambers of 0.2 ml volume and perfused at the rate of 1 ml/min with Krebs' solution (37°C) aerated with either, 21 % O_2, 5 % CO_2 and 74 % N_2 (normoxygenic buffer) or 95 % N_2 and 5 % CO_2 in the absence of glucose (hypoxia-glucopenia The base of the strip was attached to the bottom of the chamber and the apex to isometric force transducers connected to a four-channel PowerLab system (AD Instruments, Oxford, UK) running LabChart 7.0 (AD Instruments). The organ bath was modified to allow gassing of the heated water bath to reduce any gaseous exchange of oxygen through the perfusion tubes when the conditions were switched to experimental hypoxia-glucopenia. Hypoxia-glucopenia was achieved by perfusing the organ bath with glucose-free Krebs' solution gassed with 95 % N_2 and 5 % CO_2 from a sealed flask also gassed with 95 % N_2 and 5 % CO_2. Rat bladder strips were allowed to equilibrate with normoxic buffer for 1 hr under 10 mN of tension before experimentation. The bladder strips were stimulated with electrical field stimulation (EFS) using recessed platinum electrodes in the bath chamber connected to a Harvard Dual Impedance Research Stimulator capable of delivering electrical impulses at different frequencies, voltages and pulse widths. Frequency-response curves were obtained by stimulating the muscle strips with increasing frequencies,

0.5, 1, 5, 10, 20 and 40 Hz, at 50 Volts with a pulse width of 0.05 ms in 10 second trains, at 1 minute intervals after the previous response had returned to the baseline.

Electrical field stimulation and TTX

Electrical field stimulation of rat bladder muscle was used to stimulate the intramural nerves to release the co-transmitters acetylcholine (ACh) and ATP and to determine the proportion of ACh and ATP released at different frequencies of stimulation, whilst tetrodotoxin (TTX 1.6 μM; Sigma-Aldrich) was used to confirm EFS-stimulation of the intrinsic nerves without direct muscle stimulation (16). To obtain control frequency response curves, bladder strips were perfused with Krebs' solution containing 1 μM or atropine (Sigma-Aldrich) or 10 μM atropine for 20 minutes, before repeat frequency response curves were obtained. The higher atropine concentration was used in hypoxia-glucopenia and reoxygenation experiments. Further incubations with 10 μM atropine, plus 10 μM α, β-methylene-ATP, and then with TTX were performed followed by measurement of frequency response curves after each incubation period.

Effect of different oxygen concentrations on contraction

Because bladder smooth muscle *in vivo* is not exposed to high oxygen tensions, the effect of gassing the Krebs' solution, perfusing the organ bath, with either 21 % O_2, 5 % CO_2 and balance 74 % N_2 or 95 % O_2 and 5 % CO_2 on rat bladder strip contractile response was investigated. Control contractile response to 10 Hz EFS was performed when the bladder strips were perfused with Krebs' solution gassed with 21 % O_2, 5 % CO_2 and balance 74 % N_2. The gas was then switched to 95 % O_2 and

5 % CO_2 and contractile responses obtained at time points, 0, 5, 20, 40, 60, 90, 120, 180 and 240 min.

Stability of tissue response to EFS over time

Given the apparent inhibitory effect of 95 % O_2 on detrusor contractility, compared to contractile responses obtained in bladder strips gassed with 21 % O_2 (see Results section), and that tissue *in situ* would be exposed to oxygen tensions much lower than 95 %, 21 % O_2 was chosen to represent normoxia in this study. To demonstrate that bladder tissue response remained stable throughout, a series of time course experiments with rat bladder strips perfused with Krebs' solution gassed with 21 % O_2, 5 % CO_2 and balance 74 % N_2 were performed over 240 minutes with frequency response curves obtained at 0, 5, 20, 40, 60, 90, 120, 180 and 240 min.

Evoked contractile responses during hypoxia-glucopenia/reoxygenation cycling

Four cycles of hypoxia-glucopenia each lasting 10 minutes, followed by reoxygenation (normoxygenic) with glucose containing Krebs' solution for one hour were performed. Contractile responses to nerve stimulation were obtained by applying a 1 Hz electrical field stimulation to rat bladder strips before (control) and then after each period of hypoxia-glucopenia and reoxygenation. A time bar cartoon of the protocols used in this study is provided in Fig 1.

The effect of atropine or α, β, methylene-ATP on contractile responses

The effect of hypoxia-glucopenia and reoxygenation cycling on the purinergic and cholinergic components of the nerve mediated contraction were determined after each period of hypoxia-glucopenia/reoxygenation cycling by recording the bladder

contractile responses to EFS 1 Hz in the presence of 1 μM atropine or EFS 10 Hz plus 10 μM α, β-methylene-ATP (ABMA, Sigma Aldrich), respectively. The proportion of ATP to acetylcholine release varies with stimulation frequency; the effect on contractile response of ABMA desensitization is greatest at low frequencies, while the proportion of response blocked by atropine is greater at higher frequencies (Brading & Williams 1990). Stimulation frequencies of 1 and 10 Hz were selected, where the proportion of purinergic and cholinergic signaling was greater, respectively, to permit each to be most easily dissected. Tissues were subjected to two or three repeat stimulations after each period of hypoxia-glucopenia and reoxygenation to determine the effect of repeated stimulations under these conditions, which prolonged slightly the hypoxia-glucopenia and reoxygenation times.

Effects of Hypoxia-glucopenia/reoxygenation cycling on KCl responses

To determine the effect of hypoxia-glucopenia and reoxygenation cycles on a receptor independent mechanism of bladder muscle cell membrane depolarization, bladder strips were stimulated with 60 mM KCl before and after each period of hypoxia-glucopenia and reoxygenation for four cycles.

Effects of Hypoxia-glucopenia/reoxygenation cycling on agonist concentration response curves

To address the possibility of receptor desensitisation by repetitive exposure of the bladder strips to agonists after each period of hypoxia-glucopenia and reoxygenation, concentration response curves to ABMA and carbachol were performed before and after four cycles of hypoxia-glucopenia and reoxygenation. For

ABMA, bladder strips were stimulated with concentrations of 100 nM to 1 mM and for carbachol with 10 nM to 1mM.

Hypoxia-glucopenia*/reoxygenation cycling in the presence of purinoceptor antagonists*

Rat bladder contractile responses to 1 Hz EFS before and during 4 cycles of hypoxia-glucopenia and reoxygenation were repeated, as described above, in the presence of either 100 μM of the non-selective P2 receptor antagonist suramin (Tocris Bioscience, Bristol, UK), 10 μM of P2X receptor antagonist, pyridoxalphosphate-6-azophenyl-2', 5'-disulfonic acid tetrasodium salt; (*iso*-PPADS, Tocris Bioscience, Bristol, UK) or 1 μM of the selective P2X1 antagonist, pyridoxal-5'-phosphate-6-(2'-naphthylazo-6'-nitro-4', 8'-disulfonate; (PPNDS, Tocris Bioscience, Bristol, UK).

Adenosine-5'-triphosphate and acetylcholine release from bladder strips during hypoxia-glucopenia/reoxygenation cycling

Rat urinary bladder strips were weighed before being mounted in an organ bath chamber and perfused with oxygenated Krebs' solution. After 1 hour equilibration, 2 ml of the perfusate was collected from the organ bath chamber outflow and used for measurements of basal release ATP and ACh using commercial assay kits (Biaffin GmbH, Kassel, Germany, Life Technologies, Paisley, UK and Amplex Red Acetylcholine kit, A12217, respectively). Further 2 ml aliquots were then taken during stimulation (control response) and after each period of hypoxia-glucopenia and re-oxygenation. For ATP determination, 50 μl aliquots, of the perfusate were added to 50 μl of reaction buffer (prepared in the dark as instructed by the manufacturer) and

samples assayed according to the manufacturer's instructions, with the exception that a standard curve was generated over the range 1 pmol - 1000 pmol per assay in Krebs' solution. Luminescence was measured in a luminometer (Turner Biosystems model TD 20/20, Madison, USA) and quantification of ATP levels calculated by interpolation of the unknowns from the standard curve. Data are expressed as pmol ATP per mg of tissue per minute.

ACh release was determined using the Amplex Red Acetylcholine kit. Briefly, a working solution was prepared containing 400 µM Amplex red, 2 U/ml of horseradish peroxidase, 0.2 U/ml choline oxidase (from *Alcaligenes* sp) and 1 U/ml acetylcholinesterase. A standard curve of acetylcholine concentrations (from 0 to 100 µM) was created in Krebs' solution and a positive control prepared by making a 10 µM solution of stabilized hydrogen peroxide. All standards, controls and samples (100 µl), were placed into a 96 well plate. The reaction was started by adding 100 µl of working solution into each well. After 30 minutes, the plate was read on a CytoFluor Series 4000 (PerSeptive Biosystems) fluorimeter with excitation set at 530 – 560 nm and emission recorded at 590 nm.

Tissue processing and Immunofluorescence

After various experiments (above), bladder strips were removed from the organ bath and incubated overnight in 10 % neutral-buffered formalin. Samples were then processed into paraffin with tissue placed vertically in a block in such a way that tissue orientation allowed 5 µm sections to be cut so that sections contained both muscle and urothelium. Normal, whole, rat bladders were fixed and prepared in paraffin blocks in a similar way.

Rat bladder sections were deparaffinised in xylene thrice, and hydrated through 99 % IMS, 95 % industrial methylated spirit and ultrapure water for 3 minute each. Tissues were then washed with Tris Buffered Saline (TBS)-Tween (0.05 %v/v) for 5 minutes and blocked for 30 minutes in 10 % normal goat serum (NGS) in TBS-Tween (0.05 % v/v). Sections were then incubated with $P2X_1$ antibody (Millipore, UK) (validated by Vial & Evans, 2000) diluted 1:50 in blocking solution overnight in a humid chamber at 4 °C. Control samples were incubated with a 1:50 dilution of P2X1 antibody pre-incubated (w/w, 37°C, 1 h) with purified immunizing peptide (Millipore, UK). The next day, sections were washed in TBS-Tween (0.05%v/v) and incubated at room temperature with goat anti-rabbit antibody conjugated to FITC (Sigma-Aldrich, UK), 1:160 dilution in 10 % NGS for 1 hr in the dark. After washing again with TBS-Tween (0.05 % v/v) for 5 minutes, sections were counterstained with a 1:50 dilution of 4',6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) for 5 minutes. The sections were mounted with Vectorshield fluorescent mounting media (Vector Laboratories, Peterborough, UK) and the coverslips sealed with nail polish to prevent mountant drying and movement whilst being viewed under the microscope. Tissue section images were obtained using a Nikon C1Si confocal laser scanning microscope with an x 60 oil immersion objective and analyzed using NIS-Elements (Nikon, Tokyo, Japan) software. A fluorescence intensity profile for each image was obtained by analysis of three randomly placed, rectangular areas of tissue with the following dimensions: 104 µm and 44 µm. Fluorescence values for the controls were subtracted and means of the three fields calculated.

Statistical analysis

Data are expressed as mean ± S.E.M. from six bladders. Statistical analysis was determined using repeated measures ANOVA followed by Dunnett's multiple comparison test. Statistical significance was accepted when $p<0.05$. All data processing and statistical analyses were performed using GraphPad Prism version 5 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com).

RESULTS

Bladder function

Electrical field stimulation and effect of TTX

The majority of the contractile responses of the bladder strips to EFS were abolished in the presence of 1.6 μM TTX, indicating their neurogenic origin (Fig 2). The nerve-mediated response comprises of the atropine-sensitive response (cholinergic) and the atropine-resistant response (purinergic), which is mainly desensitized by α, β methylene ATP (ABMA). The small TTX sensitive, atropine and ABMA resistant response has been shown by others to be purinergic (Kennedy *et al* 2007)

Effect of different oxygen concentrations on contraction

The contractile responses of rat bladder strips to 10 Hz EFS were significantly reduced by 18 ± 3 % ($p<0.001$) when the gas for the Krebs' solution perfusing the tissue, was changed from 21 % O_2, 5 % CO_2, 74 % N_2 to 95 % O_2, 5 % CO_2. An example of a LabChart recording for a single bladder strip is shown in Fig 3A and the mean responses of muscle strips from six different rat bladders is shown in Fig 3B. Because gassing of the bladder strips with 95 % O_2 had an inhibitory effect on muscle contraction, compared to gassing the strips with 21 % O_2, gassing with 21 % O_2, 5 % CO_2, 74 % N_2, was used for all subsequent experiments.

Stability of bladder tissue response to EFS over time

Under constant gassing with 21 % O_2, there was no significant difference in the frequency response curves obtained over 240 minutes (Fig 4A). Specifically, the

contractile responses to 1 and 10 Hz showed no significant difference over time (Fig 4B).

Evoked contractile responses during hypoxia-glucopenia/reoxygenation cycling

Rat bladder strip contractile response to 1 Hz EFS increased significantly during cycles of hypoxia-glucopenia and reoxygenation compared to control responses (Fig 5A). After the second, third and fourth reoxygenation cycle, contractile responses to EFS were significantly increased by 45 ±16 % ($p<0.01$), 45 ± 13 % ($p<0.01$) and 68 ± 21 % ($p<0.001$), respectively. Apart from the first cycle, there was a trend for contractile responses to be slightly increased after each 10 minute exposure to hypoxia-glucopenia, but these were not significantly different from control responses.

Evoked contractile responses during hypoxia-glucopenia/reoxygenation cycling in the presence of either atropine or α, β, methylene ATP

After four repeated cycles of hypoxia-glucopenia and reoxygenation there was a significant increase in the atropine-resistant contractile response to 1 Hz EFS compared to the control response (Fig. 5B). After the second and fourth cycles of reoxygenation, the first atropine-resistant response to field stimulation significantly increased by 26 ± 5 % ($P<0.001$) and 40 ± 7 % ($p<0.001$), respectively, when compared to control. During the periods of hypoxia-glucopenia, the atropine-resistant response was increased by 30 ± 5 % ($p<0.001$) after the third cycle and by 28 ± 7 % ($p<0.001$) after the fourth cycle of hypoxia-glucopenia.

The contractile responses to EFS 10 Hz in the presence of α, β-methylene-ATP did not significantly change during the three reoxygenation cycles (Fig 5C). During

periods of hypoxia-glucopenia, successive α, β-methylene-ATP-resistant contractile responses were significantly reduced, with the third stimulation during each cycle being reduced more than the first. However, at the end of the three cycles of hypoxia-glucopenia and reoxygenation, the contractile responses to each of the three stimulations were similar to each other and controls. Reoxygenation restored contractility to control levels but no augmentation of cholinergic responses above control levels was observed. Stimulations with EFS were repeated 2-3 times at each point in the protocol to investigate whether the second or third responses were diminished compared to the first. During the hypoxia-glucopenia periods there was a gradual reduction in contractile response on repeated stimulation. This is particularly obvious in Fig 5C. No significant diminution was seen between the three responses in reoxygenated tissues.

Effects of hypoxia-glucopenia/reoxygenation cycling on KCl responses

During four hypoxia-glucopenia and reoxygenation cycles the contractile responses of rat bladder to 60 mM KCl did not change significantly (Fig 5D).

Effects of hypoxia-glucopenia/reoxygenation cycling on agonist concentration response curves

After four cycles of hypoxia-glucopenia and reoxygenation the concentration response curve to ABMA was shifted upwards compared to the response curve before cycling and the maximum contractile response was increased by 77 ± 24 % (Fig 5E).

The contractile responses of rat bladders to increasing concentrations of carbachol were not significantly changed after the strips had been exposed to four cycles of

hypoxia-glucopenia and reoxygenation compared to contractile responses before cycling (Fig 5F).

Hypoxia-glucopenia/reoxygenation cycling in the presence of purinoceptor antagonists

Control contractile responses of rat bladder strips to 1 Hz EFS were obtained in the absence and presence of suramin, iso-PPADS or PPNDS. Responses were then obtained during cycles of hypoxia-glucopenia and reoxygenation in the continued presence of one of these antagonists. The addition of 100 μM suramin to the Krebs' solution during cycles of hypoxia-glucopenia and reoxygenation blocked the gradual increase in contractile responses seen after reoxygenation in the absence of the purinergic antagonist (Fig 6A). All contractile responses to 1 Hz EFS during reoxygenation cycles were similar to the control responses, however, during the hypoxia-glucopenia cycles contractile responses were significantly reduced compared to the control response, except after the fourth cycle. In the presence of 10 μM Iso-PPADS, contractile responses to 1 Hz EFS during cycles of hypoxia-glucopenia and reoxygenation showed no consistent significant difference to the control responses, with the exception of those observed in hypoxia-glucopenia cycle 1 and reoxygenation cycle 3 (Fig 6B). Contractile responses during hypoxia-glucopenia cycle 1 were significantly decreased ($p<0.01$) compared to the corresponding control response and responses during reoxygenation cycle 3 were increased by 31 ± 16 % ($p<0.05$). The addition of 1 μM PPNDS to the Krebs' solution reduced the enhancing effect of hypoxia-glucopenia and reoxygenation cycling, seen in Fig 5B, on the contractile responses of rat bladder strips to 1 Hz EFS (Fig 6C). However, the contractions during reoxygenation cycles R3 and R4 were significantly enhanced by 20 ± 12 % ($p<0.05$) and 35 ± 11 % ($p<0.001$), respectively,

compared to control responses in PPNDS obtained before commencing hypoxia-glucopenia and reoxygenation cycles.

Adenosine-5'-triphosphate and acetylcholine and release from bladder strips during cycles of hypoxia-glucopenia and reoxygenation

ATP measured from extracts of Krebs' solution taken from the organ bath during bladder tissue stimulation with 1 Hz EFS and hypoxia-glucopenia/reoxygenation cycling, showed a trend towards increased ATP release over time (Fig 7A). When ATP concentrations were normalized to the percentage of basal release, there was a significant 51 ± 26 % increase in ATP release ($p<0.05$) during bladder muscle stimulation after hypoxia-glucopenia cycle 4 compared to the release during the control contraction (Fig 7B).

The amount of acetylcholine released from the rat bladder strips during stimulation with 10 Hz EFS did not change significantly after each of the 4 cycles of hypoxia-glucopenia and reoxygenation (Fig 7C). During hypoxia-glucopenia period 4, the release of acetylcholine was reduced by 25 ± 11 % compared to release during control contractions. Release during reoxygenation period 4 was only slightly reduced by 5 ± 4% compared to release during control contractions. . When amounts of acetylcholine released were expressed as a percentage of the basal release (Fig 7D), the reduction in acetylcholine released during periods of hypoxia-glucopenia was significantly reduced, e.g. in H4, by 29 ± 10 % compared to control levels.

Immunofluorescence

The distribution of $P2X_1$ receptor-associated immunofluorescence in normal rat bladder suggests that $P2X_1$ receptors were localized to the membrane of detrusor muscle cells (Fig 8A). A similar pattern of staining was seen in the urothelial layers, although the intensity of staining was weaker than in the muscle layers. Rat bladder strips that had been mounted in the organ bath for five hours without application of hypoxia-glucopenia and reoxygenation cycles, surprisingly showed considerably reduced $P2X_1$ expression in the muscle when compared to normal bladder muscle staining (Fig 8B). The localization of $P2X_1$ receptors in the bladder muscle had also changed from localised membranous staining to small areas of striated immunofluorescence within cells (see arrows in Fig 8A). Bladder strips that had been through four cycles of hypoxia-glucopenia and reoxygenation, showed diffuse $P2X_1$-associated fluorescence over the whole detrusor muscle area (Fig 8C). Mean fluorescence intensity for $P2X_1$ expression was significantly increased in bladder strips that had been subjected to four cycles of hypoxia-glucopenia and reoxygenation compared to the bladder strips that remained untreated in the organ bath for the duration of the experiment (Fig 8D).

DISCUSSION

During four cycles of hypoxia-glucopenia and reoxygenation, bladder muscle contractile responses to EFS were significantly increased. The atropine-resistant, purinergic contractile responses to nerve stimulation were significantly increased after four cycles of hypoxia-glucopenia and reoxygenation compared to the control contractions. The cholinergic responses, determined in the presence of α, β-methylene-ATP, showed significant reductions in contractile response during three periods of hypoxia-glucopenia, with no change in responses during reoxygenation periods. In contrast, the purinergic responses continued to increase during successive cycles and this increase occurred during both the hypoxia-glucopenia and the reoxygenation periods of each cycle. The increased purinergic mediated contractile responses during hypoxia-glucopenia and reoxygenation could be due to either increased ATP release from the intramural nerves, changes in P2X purinoceptor expression and function or reduced ectoATPase activity. The former two of these possibilities were investigated further in this study.

In the rat bladders, TTX abolished the majority of the contractile response to EFS, indicating that the responses were neuronal in origin (Brading & Williams 1990). The nerve-mediated responses were mainly due to the release of two neurotransmitters, acetylcholine and ATP. A small TTX-sensitive, atropine and α, β-methylene-ATP-resistant response was also identified, which has been reported before (Kennedy *et al.,* 2007). Kennedy *et al.* suggested that this third component was due to released ATP acting *via* a pathway independent of $P2X_1$ receptors. The proportion of neurotransmitters released in the bladder is related to the stimulation frequency of the intrinsic nerves within the tissue (Brading & Williams 1990). At low frequency, for

example 1Hz, the proportion of ATP released from the nerves is higher than that of acetylcholine. As the frequency increases, 10 Hz or more, the proportion of ATP released from the nerves compared to the release of acetylcholine reduces (Fig 2). We used 1 Hz and 10 Hz stimulation in this study because of these changes in proportion of neurotransmitter release. It is evident from these observations that normal rat bladder has a significant atropine-resistant component to nerve-mediated responses whereas normal human bladder does not (Bayliss *et al*1999). Bladders from patients with DO appear to develop an atropine-resistant component to nerve stimulation (Wu *et al* 1999), although the reason for this is not clear.

Blood flow to the bladder wall in animals has been determined during the normal cycle of bladder filling and voiding (Brading & Symes 2004). The data in pigs showed that reduced oxygen tension occurred for three minutes during micturition and that pigs with outflow obstruction had markedly reduced oxygen tension during micturition (Brading & Symes 2004). This period of hypoxia-glucopenia lasted for 12 minutes, after which it recovered during reperfusion. This raises the possibility that prolonged cycles of hypoxia-glucopenia and reperfusion in susceptible human individuals, together with, reduced arterial blood flow due to atherosclerotic changes in vessels in older people, may affect innervation to the bladder wall (Brading & Symes 2004). Decreased blood flow to the bladder and decreased bladder compliance are strongly correlated in the human male bladder (Lang *et al* 1999) and mild bladder ischaemia has been shown to induce bladder overactivity in rabbits (Azadzoi *et al* 1999), suggesting that these two events are related.

To investigate the possibility of modulation of purinergic receptor activation induced by periods of hypoxia-glucopenia and reoxygenation, purinoceptor antagonists were added to the perfusion system. Suramin (a non-selective P2 antagonist) blocked the augmentation of contractile responses to EFS during hypoxia-glucopenia and reoxygenation cycling (in the absence of atropine or α, β-methylene ATP), suggesting that this augmentation was due to increased purinoceptor signalling. Similarly, iso-PPADS (a non-selective P2X antagonist) also blocked the majority of the hypoxia-glucopenia and reoxygenation induced augmentation in EFS responses. In the presence of PPNDS (a selective $P2X_1$ antagonist) responses during the third and fourth cycles of hypoxia-glucopenia and reoxygenation were still significantly increased, although by not as much as the contractions without antagonists. These results suggest that P2X receptor mechanisms are involved in the hypoxia-glucopenia and reoxygenation augmentation of rat bladder contractile responses, but that other P2X receptors, apart from $P2X_1$, may also be involved, even though immunofluorescence imaging demonstrated a significant increase in $P2X_1$ receptor expression in rat bladder muscle after four cycles of hypoxia-glucopenia and reoxygenation, compared to the expression in time-control tissues.
The contractile responses of rat bladder to exogenously applied purinergic agonist, ABMA, were significantly increased after four cycles of hypoxia-glucopenia and reoxygenation, again suggesting an augmentation in post-synaptic purinergic mechanism, presumably P2X receptor expression.

$P2X_2$ receptors have been shown to be significantly elevated in bladders from women with idopathic detrusor overactivity (O'Reilly *et al* 2002) and $P2X_3$ and $P2X_5$ receptors were absent from varicosities in parasympathetic nerves of bladders from

adults with urge incontinence (Moore *et al* 2001) reducing presynaptic negative feedback.

Although other studies have investigated hypoxia-glucopenia and reoxygenation on isolated bladder strips (Pessina *et al* 1997,, Bratslavsky *et al* 1999,, Yoshida *et al* 200114), they have all examined total nerve-mediated responses and post-synaptic purinoceptor activation. None, to date, has determined the effect of hypoxia-glucopenia and reoxygenation cycling on the purinergic component of intrinsic nerve stimulation. It is important to study this component because over half of bladder samples from patients DO either idiopathic or secondary to outlet obstruction, have an atropine-resistant component to nerve-mediated responses, whereas in normal bladders the nerve-mediated responses are predominantly cholinergic, being abolished by atropine (Sjogren *et al* 1982,,Bayliss *et al* 1999,Wu *et al* 1999,,Yoshida *et al* 2001). Nevertheless, the generation of purinergic mediated contractions in overactive bladders was not due to altered sensitivities of the detrusor muscle to ATP or acetylcholine (Bayliss *et al* 1999). It is not known whether the purinergic responses were due to increased presynaptic neuronal release of ATP or inhibition of ATP breakdown by nucleotidases, although it has been suggested that inhibition of ectoATPases may play a role (Wu *et al* 1999). As an additional purinergic component to nerve stimulation was found in unstable bladders secondary to outlet obstruction and in those with idiopathic detrusor overactivity, an aetiopathology common to both diseases is possible.

Finally, ATP release from bladder strips during hypoxia-glucopenia and reoxygenation cycling was determined to investigate the possibility that increased ATP output contributed to augmented contractile responses during those cycles.

ATP release during hypoxia-glucopenia and reoxygenation cycling showedno significant changes, however, when amounts released were expressed as a percentage increase above basal levels, the increase in levels at cycle 4 was significantly different to control. It is difficult to interpret this finding for a number of reasons. Firstly, the very low levels of ATP that were released from the bladder strip to 1Hz EFS made it difficult to detect significant changes in ATP output. Use of higher, less physiological stimulation frequencies, such as 5 – 10 Hz EFS, would likely have increased ATP release only slightly, as suggested by contractile data (Brading & Williams 1990). Secondly, the source of the ATP detected from bladder strips during hypoxia-glucopenia and reoxygenation may not have been from stimulation of the excitatory terminals (Harvey *et al* 2002, Kumar *et al* 2004), but could have come from the urothelium after stretch (Smith *et al* 2005) or release from damaged cells (Wang *et al* 2005), making unequivocal interpretation difficult. The release of ACh from the bladder strips during hypoxia-glucopenia and reoxygenation did not change significantly during the reoxygenation periods but was significantly reduced during the hypoxia-glucopenia periods, however, the contractile response to exogenously applied carbachol did not change after four cycles of hypoxia-glucopenia and reoxygenation suggesting cholinergic mechanisms do not play a role in the augmentation of contractile responses of rat bladder to four cycles of hypoxia-glucopenia and reoxygenation.

Conclusions

The data presented here suggest that *in vitro* cycles of hypoxia-glucopenia and reoxygenation augment bladder muscle contraction *via* purinergic mechanisms

predominantly. These mechanisms likely include both modulation of $P2X_1$ receptor expression and ATP release. It is, therefore, possible that hypoxia-glucopenia and reperfusion episodes during the micturition cycle and atherosclerotic changes in the elderly contribute to increased atropine-resistant contractile responses seen in bladders of patients with DO, OAB and BOO. Treatment with purinergic antagonists may, therefore, be a useful option.

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Figure legends

Fig 1. A time bar cartoon showing the basic protocol used in this study. E is the equilibration time (60 minutes) for the bladder strips before commencing electrical field stimulation (downward arrows). In protocols with drug addition, these were added during the last 20 minutes of the equilibration time and were present throughout the four cycles of hypoxia-glucopenia and reoxygenation. H is the period of hypoxia-glucopenia and R the period of reoxygenation. At the end of each period of hypoxyia-glucopenia and of reoxygenation, two trains (10 seconds) of electrical field stimulation were applied separated by 1 minute (shown by double set of downward pointing arrows).

Fig 2. Contractile responses of isolated rat detrusor muscle to electrical field stimulation in the absence and presence of cholinergic and purinergic antagonists. ○ Control contractile response (57 ± 8.0 mN at 20 Hz), contractile responses after the addition of △ atropine 1 μM, ▽ atropine 10 μ M, □ atropine 10 μM plus α, ß-methylene-ATP 10 μM, ◇TTX 1.6 μM. n = 6.

Fig 3. The effect of changing the Krebs' solution gassed with 21 % O_2 to Krebs' solution gassed with 95 % O_2 on isolated rat bladder muscle contractile response to electrical field stimulation.

(A) Representative LabChart recording of frequency response recordings (1, 5, 10, 20, 40 Hz), the results of which were included in the construction of the graph in panel B. (B) Contractile responses to 10 Hz EFS after in perfused Krebs' solution gassed with 21 % O_2, 5 % CO_2 and balance nitrogen and after switching to Krebs'

solution gassed with 95% O_2 and 5% CO_2 (arrow). ** p< 0.01, *** p< 0.001 (Repeated measures ANOVA followed by Dunnett's multiple comparison test; n = 6).

Fig 4.Contractile responses of isolated bladder muscle to EFS over time. Repeat frequency response curves at 0 ● , 5 □, 20 △, 40 ▽, 60 ◊, 90 ○, 120 ■, 180 ▲ and 240 ▼ min (A). Contractile responses to 1Hz ○ and 10 Hz △ over 240 minutes expressed as % maximum response at 20 Hz (B). Data are from n = 6 experiments.

Fig 5. The effect of hypoxia-glucopenia and reoxygenation cycling on the contractile responses of rat detrusor muscle to repeated electrical field stimulation and stimulation with KCl, ABMA and carbachol. (A-C) Contractile responses of rat detrusor muscle to 1 Hz EFS during 3 – 4 cycles of hypoxia-glucopenia (H1 – H4, grey bars) (10 min) and reoxygenation (R1 –R4, white bars) (1 hour). Repeat **c**ontractile responses to 1Hz EFS were measured after each hypoxia-glucopenia and reoxygenation cycle (A-C), Contractile responses to 1Hz EFS after each hypoxia-glucopenia and reoxygenation cycle in the absence of antagonist (A). **C**ontractile responses to 1Hz EFS after each hypoxia-glucopenia and reoxygenation cycle in the presence of 1 μM atropine (B) and to 10 Hz EFS in the presence of α, β, methylene ATP 30 μM (C). Single responses to 60 mM KCl after each hypoxia-glucopenia and reoxygenation cycle (D). Concentration response curves to α, β, methylene ATP (n=5) (E) and carbachol (F) before ○ and after □ four cycles of hypoxia-glucopenia and reoxygenation
Hatched bars are the control contractile responses before commencing hypoxia-glucopenia and reoxygenation cycling. Data are the mean ± S.E.M. of 6 replicates. *

P<0.05, ** p<0.01, *** p<0.001 compared to control (Repeated measures ANOVA followed by Dunnett's multiple comparison test).

Fig 6. The effect of P2X receptor antagonists on the augmentation of detrusor muscle contractile responses to hypoxia-glucopenia and reoxygenation cycling. Contractile responses of rat detrusor muscle to 1 Hz EFS during 4 cycles of hypoxia-glucopenia (10 min, H1 – H4, grey bars) and reoxygenation (1 hour, R1 – R4, white bars) in the presence of 100 μM suramin (A) 10 μM iso-PPADS (B) or 1μM PPNDS (C). Contractile responses before commencing cycling in the presence of the purinergic antagonists are shown as hatched bars. Data are the mean ± S.E.M. of 6 replicates.
* P<0.05, ** p<0.01, *** p<0.001 (Repeated measures ANOVA followed by Dunnett's multiple comparison test).

Fig 7. ATP and acetylcholine release from bladder strips stimulated with 1 Hz EFS during 4 cycles of hypoxia-glucopenia and reoxygenation.
ATP release expressed as pmol/mg of bladder tissue wet weight/min (A) and as percent basal release (B), n= 10. Acetylcholine release expressed as pmol /mg of bladder tissue wet weight/min (C) and as percent basal release (D), n= 6. The data are the mean ± S.E.M.* P<0.05, ** p<0.01, *** p<0.001 (Repeated measures ANOVA followed by Dunnett's multiple comparison test).

Fig 8. Immunofluorescence staining for $P2X_1$ receptor expression in rat bladder muscle before and after exposure to four cycles of hypoxia-glucopenia and reoxygenation.

Normal rat bladder sample expressing $P2X_1$ receptor (green fluorescence, n=2) (A); $P2X_1$ receptor expression in time-controlled rat bladder that was incubated in the organ bath for 240 minutes but not exposed to hypoxia-glucopenia and reoxygenation, nuclei staining (DAPI) is merged to demonstrate the presence of cells not expressing $P2X_1$ receptors (B). (C) $P2X_1$ receptor expression in rat bladder tissue after exposure to 4 cycles of hypoxia-glucopenia and reoxygenation (D). Fluorescence intensities of $P2X_1$ expression in fresh rat bladder and in time control bladder tissue (without H/R) compared to tissue that has been exposed to hypoxia-glucopenia/reoxygenation (with H/R). n=3 *$p<0.05$, ** $p<0.01$ (Student's t test)