(margin, left) **The Journal of PHARMACOLOGY And Experimental Therapeutics**

Activation of Prejunctional P2x2/3 Heterotrimers by ATP Enhances the Cholinergic Tone in Obstructed Human Urinary Bladders

M. Silva-Ramos,[1] I. Silva,[1] M. Faria,[2] F. Ferreirinha, and P. Correia-de-Sá

Laboratório de Farmacologia e Neurobiologia and Center for Drug Discovery and Innovative Medicines (MedInUP), Instituto de Ciências Biomédicas de Abel Salazar (ICBAS), Universidade do Porto, Porto, Portugal (M.S.-R., I.S., M.F., F.F., P.C.-S.); and Serviço de Urologia, Centro Hospitalar Universitário do Porto (CHUP), Porto, Portugal (M.S.-R.)

ABSTRACT

The objective of this study was to investigate the role of ATP in cholinergic neurotransmission in the urinary bladder of control men and of patients obstructed as a result of benign prostatic hyperplasia (BPH). Human detrusor samples were collected from 41 patients who submitted to transvesical prostatectomy resulting from BPH and 26 male organ donors. The release of [³H]acetylcholine ([³H]ACh) was evoked by electrical field stimulation (10 Hz, 200 pulses) in urothelium-denuded detrusor strips. Myographic recordings were performed to test detrusor strip sensitivity to ACh and ATP. Nerve-evoked [³H]ACh release was 1.5-fold higher in detrusor strips from BPH patients compared with controls. This difference was abolished after desensitization of ionotropic P2X1-3 receptors with an ATP analog, α,β-methylene ATP (30 μM, applied for 15 minutes). TNP-ATP (10 nM, a preferential P2X2/3 antagonist) and A317491 (100 nM, a selective P2X3 antagonist) were about equipotent in decreasing nerve-evoked [³H]ACh release in control detrusor strips, but the selective P2X1 receptor antagonist NF023 (3 μM) was devoid of effect. The inhibitory effect of TNP-ATP (10 nM) increased from 27% ± 9% to 43% ± 6% in detrusor strips of BPH patients, but the effect of A317491 (100 nM) [³H]ACh release

unaltered (20% ± 2% vs. 24% ± 4%). The amplitude of ACh (0.1–100 μM)-induced myographic recordings decreased, whereas sensitivity to ATP (0.01–3 mM) increased in detrusor strips from BPH patients. Besides the well characterized P2X1 receptor-mediated contractile activity of ATP in pathologic human bladders, we show here for the first time that cholinergic hyperactivity in the detrusor of BPH patients is facilitated by activation of ATP-sensitive P2X2/3 heterotrimers.

SIGNIFICANCE STATEMENT

Bladder outlet obstruction often leads to detrusor overactivity and reduced bladder compliance in parallel to atropine-resistant increased purinergic tone. Our data show that P2X1 purinoceptors are overexpressed in the detrusor of patients with benign prostatic hyperplasia. Besides the P2X1 receptor-mediated detrusor contractions, ATP favors nerve-evoked acetylcholine release via the activation of prejunctional P2X2/3 excitatory receptors in these patients Thus, our hypothesis is that manipulation of the purinergic tone may be therapeutically useful to counteract cholinergic overstimulation in obstructed patients.

Introduction

Bladder outlet obstruction (BOO) often leads to detrusor overactivity (DO) and reduced bladder compliance causing urine storage symptoms (Mirone et al., 2007). DO is detected in about half to two-thirds of patients with diagnosed benign prostatic hyperplasia (BPH) and has a huge impact on patients' quality of life (Mirone et al., 2007). The prevalence of DO decreases by 50% after prostatic surgery (Abrams et al., 1979), suggesting that it may be caused, at least in part, by BOO. Notwithstanding this, the mechanisms underlying DO caused by obstruction are largely unknown (Andersson, 2003). On the other hand, subsequent failure of the detrusor to

ABBREVIATIONS: A317491, 5-[[[(3-phenoxyphenyl) methyl] [(1S)-1,2,3,4-tetrahydro-1-naphthalenyl] amino] carbonyl]-1,2,4-benzenetricarboxylic acid sodium salt hydrate; ARL67156, 6-N,N-diethyl-D-β,γ-dibromomethylene ATP trisodium salt; BOO, bladder outlet obstruction; BPH, benign prostatic hyperplasia; DAPI, 4′,6-diamidino-2-phenylindole; DO, detrusor overactivity; DU, detrusor underactivity; EFS, electrical field stimulation; [³H]ACh, [³H]acetylcholine; α,β-MeATP, α,β-methylene ATP; NF023, 8,8′-[carbonylbis(imino-3,1-phenylenecarbonylimino)]bis-1,3,5-naphthalenetrisulphonic acid hexasodium salt; NTPDases, ecto-nucleoside triphosphate diphosphohydrolases; PPADS, pyridoxal phosphate-6-azo (benzene-2,4-disulfonic acid) tetrasodium salt hydrate; TNP-ATP, 2′,3′-O-(2,4,6-trinitrophenyl)adenosine-5′-triphosphate tetra(triethylammonium) salt.

contract may appear at a later stage of obstructed bladder dysfunction. Detrusor underactivity (DU) is defined by the International Continence Society as a contraction of reduced strength and/or duration resulting in prolonged and incomplete emptying of the bladder. Although a bit forgotten from the scientific literature, this aspect of the bladder physiology is gaining increased attention (van Koeveringe et al., 2011). DU has been associated with age (Van Mastrigt and Rollema, 1992) and its prevalence is thought to be increasing owing to ageing of the population. Until now, the basis of treatment has been bladder catheterization and no effective therapeutic drug is yet available.

Increasing evidence supports a role for purines in the pathophysiology of DO and in bladder changes resulting from BOO (Burnstock, 2011). In humans, normal detrusor contraction is exclusively under cholinergic control, since it is fully blocked by atropine (Fry et al., 1999). However, in hypertrophic bladders secondary to prostatic obstruction atropine-resistant contractions appear (Sjögren et al., 1982; Bayliss et al., 1999), and they have been attributed to ATP because they are fully blocked by desensitization of ionotropic P2X receptors with the enzymatically stable ATP analog α,β-methylene ATP (Bayliss et al., 1999). However, there are gaps in our knowledge regarding the purinergic control of the bladder function, mostly because the balance between the cholinergic and purinergic components varies significantly among species (Wust et al., 2002; Ford et al., 2006) and, thus, when comparing rodents to humans, the observed changes in response to bladder obstruction are unpredictable. For instance, the ATP-mediated component is already relevant in normal bladder contractions in rodents and rabbits, but it is not exaggerated in obstructed (Banks et al., 2006) and neurogenic (Yokota and Yamaguchi, 1996) bladders, rendering these models less suitable for studying (purinergic) detrusor changes after BOO.

The prominent role of ATP in the bladder of men with BPH has been essentially attributed to its ability to increase detrusor smooth muscle tension (Bayliss et al., 1999). The mechanism underlying the increased ATP activity in the bladders of obstructed patients has been attributed to decreased ecto-ATPase activity (Harvey et al., 2002) leading to reduced inactivation of the nucleotide and, thus, increased lifetime of ATP released by the urothelium and cholinergic bladder efferents (Silva-Ramos et al., 2015). In addition, our group demonstrated that the release of ATP is also higher in urothelium-denuded detrusor strips from BPH patients compared with age-matched controls (Silva-Ramos et al., 2010, 2015).

Besides its action as a cotransmitter in several myoneural junctions, ATP can also act as a neuromodulator controlling the release of acetylcholine (ACh) and other neurotransmitters (Ribeiro et al., 1996; Duarte-Araújo et al., 2009; Barros-Barbosa et al., 2016, 2018; Vieira et al., 2017). The neuromodulatory role of ATP has been demonstrated in the bladders of rats and pigs (King et al., 1997; D'Agostino et al., 2012). ATP favored cholinergic neurotransmission in the pig detrusor via the activation of presynaptic P2X3 receptors, and the facilitatory role of the nucleotide was further enhanced by the ecto-ATPase inhibitor ARL67156 (D'Agostino et al., 2012). Recently, we showed that pathologic deficits in ATP inactivation are observed in the bladder of BPH patients, leading to deficient extracellular adenosine formation and cholinergic hyperactivity as a consequence of a loss of A_1-receptor-mediated neural inhibitory tone (Silva-Ramos et al., 2015).

To our knowledge, no study has so far been undertaken to explore the presynaptic repercussion of surplus ATP accumulation on ACh release from cholinergic nerves in obstructed human detrusor samples compared with individuals with normal bladder function. The results obtained may provide novel targets for therapeutic intervention in obstructed patients with detrusor overactivity and/or underactivity.

Materials and Methods

Human Detrusor Samples. Samples of human detrusor were collected from the bladder domes of 41 patients with BOO owing to BPH during transvesical prostatectomy (aged 71 ± 7 years) and from 26 male cadaveric organ donors without known lower urinary tract pathology (aged 50 ± 17 years). BOO and prostate enlargement were confirmed by uroflowmetry and ultrasonography, respectively. Collected samples were immediately placed at 4–6°C in mannitol transplantation solution at 400 mOsm/kg (M-400) not supplemented with ATP or adenosine (230 mM mannitol, 15 mM KH_2PO_4, 43 mM $K_2HPO_4\cdot3H_2O$, 15 mM KCl, and 10 mM $NaHCO_3$, pH 7.4) and transported to the laboratory. Experiments were performed within the first 24 hours after collection, which corresponds to the tissue viability window. This study and all its procedures were approved by the Ethics Committees of CHUP and ICBAS-UP and were authorized by the National Transplantation Committee. All BPH patients signed an informed consent approved by the Ethics Committee of CHUP for use of the biologic material. Regarding deceased organ donation, the legal frame work allows the "Presumed Consent," stating that residents in Portugal are consenting donors unless the individual previously objected during his or her life. The investigation conformed to the principles outlined in *The Code of Ethics of the World Medical Association* (Declaration of Helsinki).

Quantification of [³H]ACh Release. Human detrusor strips (approx. 1.5×5 mm) were mounted in vertical superfusion organ baths. The procedures used for labeling the preparations and measuring evoked [³H]ACh release followed the previously described protocol with minor modifications (Silva-Ramos et al., 2015; Silva et al., 2017). After a 30-minute equilibration period, cholinergic nerve terminals were labeled for 40 minutes with 1 mM [³H]-choline (specific activity 2.5 mCi·nmol^{-1}) under electrical stimulation (EFS, 1-Hz frequency, 1-millisecond pulse width). Washout of the preparations was performed during 60 minutes, by superfusion (15 ml minute^{-1}) with Tyrode's solution supplemented with choline uptake inhibitor hemicholinium-3 (10 μmol·l^{-1}). Tritium outflow was evaluated by liquid scintillation spectrometry (TriCarb2900 TR; Perkin Elmer, Boston) (% counting efficiency: 55% $\pm$ 2%) after appropriate background subtraction, using 400-μl bath samples collected automatically every 3 minutes with a fraction collector (FC203B; Gilson, France). [³H]ACh release was evoked by two periods of EFS (S_1 and S_2, 200 pulses of 0.5-millisecond duration delivered at 10-Hz frequency). Therefore, the evoked [³H]ACh release was calculated by subtracting the basal tritium outflow from the total tritium outflow during the stimulation period (see e.g., Carneiro et al., 2014; Silva-Ramos et al., 2015; Silva et al., 2017).

Test drugs were added 15 minutes before S_2 and were present until the end of the experiments. The change in the ratio between the evoked [³H]ACh release during the two stimulation periods (S_2/S_1) relative to that observed in control situations (in the absence of test drugs) was taken as a measure of the effect of the tested drugs.

Myographic Recordings. Detrusor muscle strips without the mucosa were mounted in 10-ml capacity perfusion chambers connected to isometric force transducers. The changes in tension were recorded continuously with a PowerLab data acquisition system (Chart 5, v.4.2; AD Instruments). Tissues were preloaded with 5 mN of tension and allowed to equilibrate for 90 minutes in Tyrode's solution, at 37°C. Contractile responses were elicited by cumulative applications of ACh (0.1–100 μM) or ATP (0.01–3 mM). Nerve-evoked contractile responses elicited by EFS trains of 5-second duration delivered every 2 minutes at increasing

frequencies (1–50 Hz, 1-millisecond pulse width) were also recorded in detrusor strips from control individuals and BPH patients; the magnitude of EFS-evoked detrusor contractions was normalized by the myographic response produced by high extracellular KCl (60 mM) in the same preparation. Dependence of EFS-induced detrusor contractile responses on generation of nerve action potentials was confirmed by blockage with tetrodotoxin (1 μM) (Fig. 1, B and C).

Immunofluorescence Staining and Confocal Microscopy Observation. Detrusor samples fragments were fixed in PLP solution (paraformaldehyde 2%, lysine 0.075 M, sodium phosphate 0.037 M, sodium periodate 0.01 M) for 16 hours at 4°C. Fixed tissue was cryoprotected with a solution containing 20% anhydrous glycerol dissolved in 0.1 M phosphate buffer, frozen, sectioned (16-μm), and incubated with a blocking buffer solution consisting of fetal bovine serum 10%, bovine serum albumin 1%, Triton X-100 0.3% in phosphate-buffered saline (PBS), for 2 hours with constant stirring. After blocking and

permeabilization, samples were incubated with rabbit anti-P2X1 primary antibody (1:75; #APR-001; Alomone, Jerusalem, Israel) diluted in the incubation buffer (fetal bovine serum 5%, serum albumin 1%, Triton X-100 0.3% in PBS), at 4°C, for 16 hours. After unbound primary antibody was washed away, the sections were incubated with donkey anti-rabbit IgG secondary antibody (1:1000; Alexa Fluor 488, #A-21206; Invitrogen) in the dark for 2 hours, at room temperature. Finally, tissue samples were mounted on optical-quality glass slides using VectaShield with DAPI as mounting medium (VectorLabs) and stored in the dark at 4°C. Observations were performed and analyzed with a laser-scanning confocal microscope (FluoView, FV1000; Olympus, Tokyo, Japan). Immunofluorescence images were quantified according to those described by Vieira et al. (2017) with minor modifications. Images were stored in TIFF format with the same resolution and subsequently analyzed with the ImageJ software version 1.46r (National Institutes of Health) to quantify the density of stained
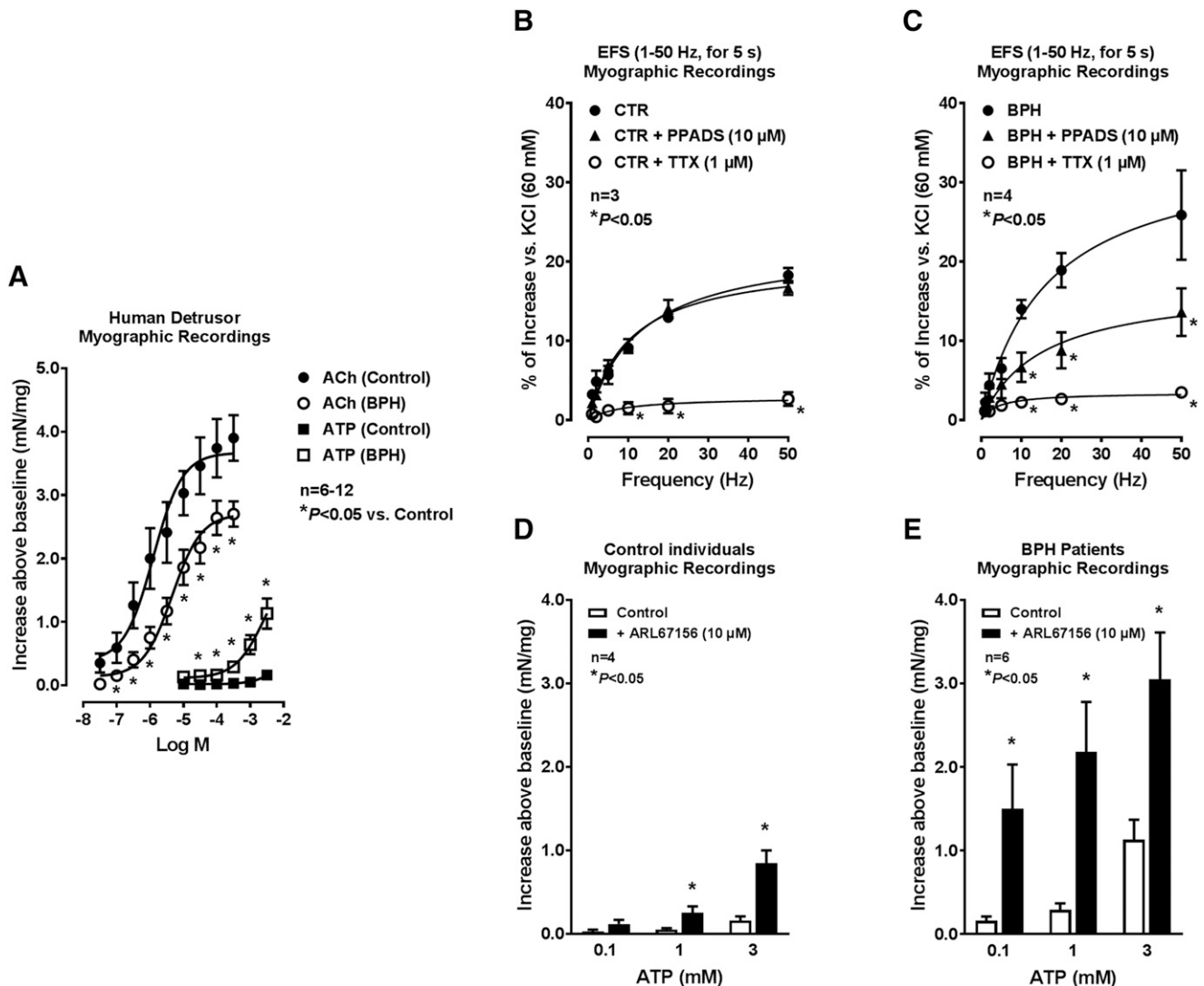
Fig. 1. (A) Contractile responses of isolated human detrusor strips from control individuals and BPH patients to ACh (0.03–300 μM) and ATP (0.01–3 mM). The magnitude of nerve-evoked detrusor contractions from control individuals (B) and BPH patients (C) in the absence and in the presence of the nerve action potentials blocker tetrodotoxin (TTX, 1 μM), or of the nonselective P2 purinoceptor antagonist PPADS (10 μM) are also shown for comparison. Detrusor strips were stimulated with EFS trains of 5-second duration at increasing frequencies (1–50 Hz, 2-minute intertrain intervals). Drugs were added 15 minutes before starting the stimulation protocol. The magnitude of EFS-evoked detrusor contractions was normalized by the myographic response produced by high extracellular KCl (60 mM) in the same preparation. The vertical bars represent S.D. of an n number of individuals and are shown when they exceed the symbol in size. *$P < 0.05$ (unpaired Student's t-test corrected for multiple comparisons using the Holm-Sidak method) represent significant differences compared with control organ donors (in A) or to the absence of test drugs (in B and C), respectively. Pretreatment with the NTPDase inhibitor ARL67156 (100 μM) potentiates ATP (0.1–3 mM)-induced contractions of detrusor strips isolated from control individuals (D) and BPH patients (E). Bars represent mean $\pm$ S.D. of six individuals. *$P < 0.05$ (unpaired Student's t-test corrected for multiple comparisons using the Holm-Sidak method) represent significant differences compared with the effect of ATP alone.

P2X1 immunoreactivity of the human detrusor. Settings such as the area, the integrated density, and the mean gray value were measured systematically in all analyzed images; the background setting was the primary antibody. The values obtained were used to calculate the corrected total cryosection fluorescence (CTCF) using a formula published in http://sciencetechnblog.com/2011/05/24/measuring-cell-fluorescence-using-imagejhttps://sciencetechblog.wordpress.com/2011/05/24/measuring-cell-fluorescence-using-imagej/.

Western Blot Analysis. Human detrusor strips without the mucosa were homogenized in radioimmunoprecipitation assay buffer (Tris-HCl 25 mM, pH 7.6, NaCl 150 mM, sodium deoxycholate 1%, Triton-X-100 1%, SDS 0.1%, EDTA 5 mM) plus a protease inhibitor cocktail. Protein content of the samples was evaluated using the BCA protein assay kit according to the manufacturer's instructions (Pierce, Rockford, IL). Samples were solubilized in SDS reducing buffer (Tris-HCl 125 mM, pH 6.8, SDS 4%, bromophenol blue 0.005%, glycerol 20%, and 2-mercaptoethanol 5%) at 70°C for 10 minutes, subjected to electrophoresis in 10% SDS-polyacrylamide gels, and electrotransferred onto polyvinylidene difluoride membranes (MilliPore, MA). Protein load was 65 μg. The membranes were then blocked in Tris-buffered saline (in millimolars: Tris-HCl 10, pH 7.6, NaCl 150) containing Tween 20 0.05% and bovine serum albumin 5%, and subsequently incubated with P2X1 primary antibody from Alomone (host species: rabbit, 1:200, APR-001) in the above blocking buffer overnight at 4°C. Membranes were washed three times for 10 minutes in Tris-buffered saline/Tween-20 and incubated with horseradish peroxidase–conjugated secondary antibody (1:25,000; Abcam, Cambridge, UK) for 1 hour at room temperature. For normalization purpose, membranes were incubated with mouse anti-GAPDH (EC 1.2.1.12) primary antibody from Santa Cruz (1:200, SC-32233; Dallas, TX) following the procedures described above. Membranes were washed three times for 10 minutes, and the antigen-antibody complexes were visualized with the Immun-Star WesternC Chemiluminescent Kit using the ChemiDoc MP imaging system (Bio-Rad Laboratories, Hercules, CA).

Drugs and Solutions. Hemicholinium-3, choline chloride, and pyridoxal phosphate-6-azo (benzene-2,4-disulfonic acid) tetrasodium salt hydrate (PPADS) were from Sigma (St. Louis, MO); ATP, α,β-methylene ATP, 2′,3′-O-(2,4,6-trinitrophenyl)adenosine-5′-triphosphate tetra(triethylammonium) salt (TNP-ATP), 6-N,N-diethyl-D-β,γ-dibromomethylene ATP trisodium salt (ARL67156), 5-[[[(3-phenoxyphenyl) methyl] [(1S)-1,2,3,4-tetrahydro-1-naphthalenyl] amino] carbonyl]-1,2,4-benzenetricarboxylic acid sodium salt hydrate (A317491), 8,8′-[carbonylbis(imino-3,1-phenylenecarbonylimino)]bis-1,3,5-naphthalene-trisulphonic acid hexasodium salt (NF023), and tetrodotoxin were obtained from Tocris Bioscience (Bristol, UK); [methyl³H]choline chloride (in ethanol, 85.5 Ci.mmol⁻¹) was from Perkin Elmer; PPADS was made up as 3 mmol·l⁻¹, and TNP-ATP was made up as 10 mmol·l⁻¹ stock solution in distilled water. A317491 was prepared in dimethyl sulfoxide. All the other compounds were dissolved in Tyrode's solution. PPADS was kept protected from light to prevent photodecomposition. All stock solutions were stored as frozen aliquots at −20°C. Dilutions of these stock solutions were made daily and appropriate solvent controls were done. No statistically significant differences between control experiments made in the absence or in the presence of the solvents at the maximal concentrations used (0.5% v/v) were observed.

Presentation of Data and Statistical Analysis. Results are expressed as mean ± S.D., with n indicating the number of individuals used for a particular set of experiments. Only one experimental procedure (e.g., agonist in the absence and in the presence of the antagonist) was performed per individual. Statistical analysis of data were carried out using Graph Pad Prism 7.04 for Windows software (La Jolla). Unpaired Student's t-test corrected by the Holm-Sidak method for multiple comparisons was used in most circumstances. Two-way analysis of variance (ANOVA) followed by the Sidak's multicomparison test was used only if F was significant and there was no variance inhomogeneity. Pearson r was used to test correlations. $P < 0.05$ (two-tailed) values were considered statistically significant.

Results

Contractile Responses of Urothelium-Denuded Detrusor Strips from Control Organ Donors and BPH Patients to ACh and ATP. Bath application of acetylcholine (ACh, 0.03–300 μM; LogEC$_{50}$ = −5.91) concentration-dependently increased smooth muscle tension in the detrusor of control organ donors (Fig. 1A). However, cholinergic-induced detrusor contractions (LogEC$_{50}$ = −5.34) had a smaller magnitude ($P < 0.05$) in samples from patients with BOO owing to BPH (Fig. 1A). For instance, the contractile activity of ACh (100 μM) decreased ($P < 0.05$) from 3.74 ± 0.46 ($n = 12$) to 2.64 ± 0.27 mN/mg ($n = 12$) in control individuals and BPH patients, respectively. To exclude bias owing to age difference between BPH patients (71 ± 7 years) and control organ donors (50 ± 17 years), we performed a correlation analysis between age and the magnitude of ACh (10 μM)-induced contractions; the Pearson's r value obtained was −0.048, which did not reach statistical significance ($P = 0.810$). In view of this, ageing does not seem to contribute, on its own, to decrease the sensitivity of detrusor smooth muscle fibers to ACh in obstructed bladders.

ATP (0.01–3 mM) was virtually devoid of effect on detrusor strips from control individuals (Fig. 1A), but the nucleotide concentration-dependently increased ($P < 0.05$) the contractile tension of urothelium-denuded detrusor strips isolated from BPH patients. At the maximal concentration tested, ATP (3 mM)-induced detrusor contractions reached 43% (1.13 ± 0.24 mN/mg, $n = 6$) of the effect caused by 100 μM ACh in BPH patients and this value was significantly ($P < 0.05$) higher than that observed in the strips from control individuals (0.16 ± 0.05 mN/mg, $n = 6$) (Fig. 1A). It is also worth noting that the P2 purinoceptor-mediated component of nerve-evoked detrusor contractions was more evident in preparations from BPH patients than in control individuals. This was noticed because the blocking effect of PPADS (10 μM, a nonselective P2 purinoceptor antagonist) only became apparent in detrusor strips from BPH patients (Fig. 1C), whereas this drug was devoid of effect on nerve-evoked detrusor contractions from control organ donors (Fig. 1B).

At the same concentration, PPADS (10 μM) prevented ($P < 0.05$) the contractile effect of ATP (0.03–3 mM) on detrusor strips from BPH patients. Prevention of ATP (0.03–3 mM)-induced detrusor contractions by PPADS (10 μM) was mimicked by selective blockage of the P2X1 receptor with NF023 (3 μM), but the same was not observed with TNP-ATP (10 nM, a preferential P2X2/3 antagonist) and A317491 (100 nM, a selective P2X3 antagonist) (Fig. 2).

The competitive inhibitor of human NTPDase1 (K_i 11 ± 3 μM) and NTPDase3 (K_i 18 ± 4 μM), ARL67156 (100 μM), significantly ($P < 0.05$) potentiated the contractile activity of ATP (0.1–3 mM) on detrusor strips isolated from both control individuals (Fig. 1D) and BPH patients (Fig. 1E), without affecting the magnitude of ACh (0.03–300 μM)-induced detrusor contractions (data not shown). The magnitude of ARL67156 (100 μM) potentiation of ATP-induced detrusor contractions was higher in BPH patients. Overall, these results confirm our previous assumption that NTPDase1/CD39 is mainly responsible for ATP/ADP enzymatic inactivation in the human detrusor (Silva-Ramos et al., 2015) and suggest that sensitivity to changes in the hydrolytic activity of ecto-nucleoside triphosphate
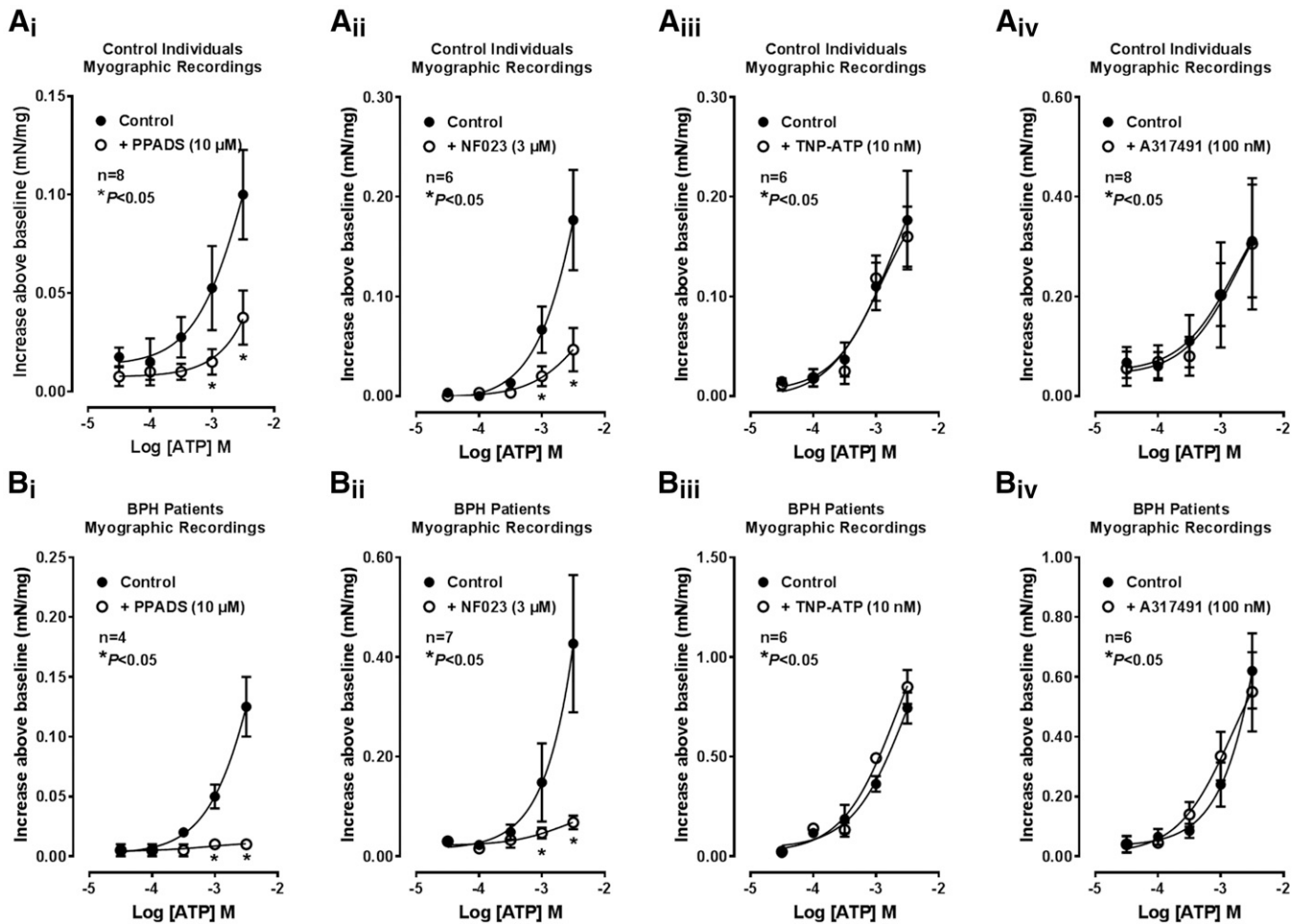
Fig. 2. ATP (0.01–3 mM)-induced contractile responses of isolated human detrusor strips from (A) control individuals and (B) BPH patients in the absence and in the presence of PPADS (10 μM), NF023 (3 μM), TNP-ATP (10 nM), and A317491 (100 nM). The vertical bars represent S.D. of an n number of individuals and are shown when they exceed the symbol in size. *$P < 0.05$ (unpaired Student's t-test corrected for multiple comparisons using the Holm-Sidak method) represent significant differences compared with the effect of ATP alone.

diphosphohydrolases (NTPDases) by ARL67156 (100 μM) has a higher impact in the detrusor of obstructed patients.

The P2X1 Receptor Is Overexpressed in Detrusor Smooth Muscle Fibers of BPH Patients Compared with Control Individuals. The presence of P2X1 receptors in the human detrusor was demonstrated by immunofluorescence confocal microscopy (Fig. 3A). The staining pattern indicates that P2X1 receptors are located on the plasma membrane of detrusor smooth muscle fibers (Fig. 3A$_i$; see also Elneil et al., 2001). Corrected total cryosection fluorescence quantification (Fig. 3A$_{ii}$) and Western blot analysis (Fig. 3B) demonstrated that P2X1 receptors were overexpressed in detrusor samples from BPH patients compared with control individuals. Overexpression of the P2X1 receptor protein was in agreement with previous RT-PCR studies (O'Reilly et al., 2001) and may explain amplification of bladder contractions caused by ATP in BPH patients compared with control individuals.

Endogenous ATP Contributes to Superfluous [³H] ACh Release in Urothelium-Denuded Detrusor Strips from BPH Patients Via the Activation of P2X2/3 Heterotrimers. Nerve-evoked [³H]ACh release after the first stimulation period (S$_1$) expressed as a percentage of the total radioactivity present in the tissue at the beginning of the collection period (fractional release, %) was 1.5-fold higher

($P < 0.05$) in urothelium-denuded detrusor strips from BPH patients (0.84 $\pm$ 0.03, $n = 6$) compared with control subjects (0.55 $\pm$ 0.02, $n = 4$) (Fig. 4; cf. Silva-Ramos et al., 2015). Interestingly, nerve-evoked contractions of detrusor strips from BPH patients (Fig. 1C) also had a higher ($P < 0.05$) magnitude than those observed in control preparations (Fig. 1B); i.e., the maximal detrusor tension elicited by 50-Hz trains normalized by the effect of high extracellular KCl (60 mM) increased ($P < 0.05$) from 18.3% $\pm$ 1.6% ($n = 3$) in control individuals to 25.9% $\pm$ 9.8% ($n = 4$) in BPH patients. A similar phenomenon was verified using 10- and 20-Hz stimulation trains.

The cholinergic hyperactivity was abrogated after desensitization of ionotropic P2X1-3 receptors with the enzymatically-stable ATP analog α,β-MeATP (30 μM); incubation with α,β-MeATP (30 μM) for 15 minutes reduced by 14% $\pm$ 6% ($n = 6$) the release of [³H]ACh from stimulated detrusor strips from control individuals, but this decrease was more evident (52% $\pm$ 7%, $n = 4$) in BPH patients (Fig. 4). A similar inhibitory response was obtained using PPADS (10 μM), which nonselectively blocks P2 purinoceptors; PPADS (10 μM) reduced nerve-evoked [³H]ACh release by 6% $\pm$ 8% ($n = 4$) and 43% $\pm$ 6% ($n = 4$) in control individuals and BPH patients, respectively (Fig. 5).
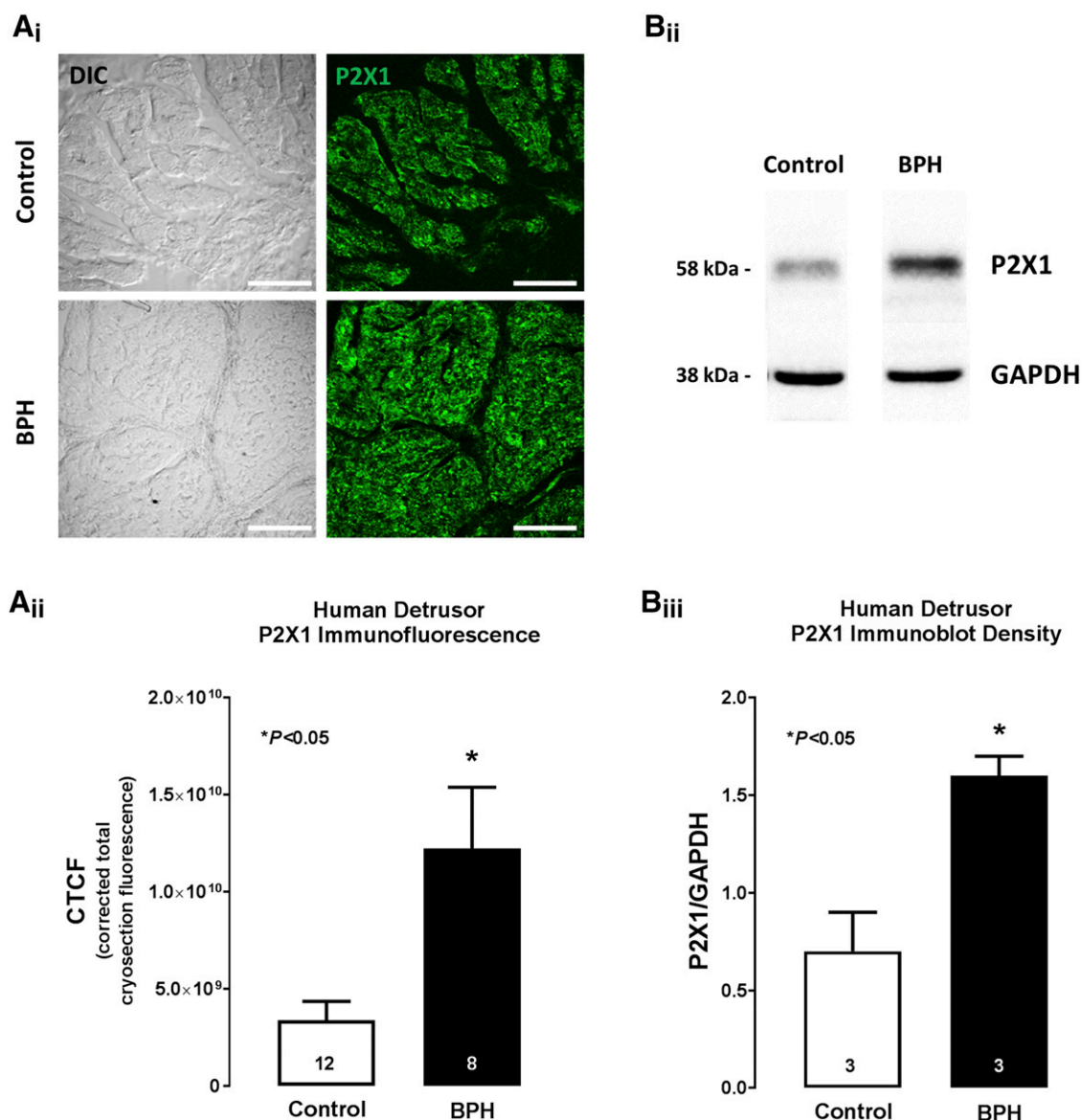
Fig. 3. The P2X1 receptor is upregulated in urothelium-denuded detrusor strips from BPH patients compared with control organ donors. (A) Confocal micrographs of transverse sections of the human detrusor isolated from control individuals and BPH patients stained with an antibody specific for a highly conserved regions of the P2X1 receptor intracellular C-terminus (APR-001; Alomone). The P2X1 receptor immunoreactivity follows a plasma membrane staining pattern. Differential interference contrast (DIC) micrographs are also shown for comparison. Scale bar, 20 μm. The bar chart shown in (A$_{ii}$) represents mean ± S.D. of corrected total cryosection fluorescence (CTCF) staining for the P2X1 receptor in the detrusor of control individuals and BPH patients. (B) Representative immunoblots of the P2X1 receptor in urothelium-denuded male detrusor homogenates of one control individual and one BPH patient run in parallel. Gels were loaded with 65 μg of protein. The bar chart shown in (B$_{ii}$) represents mean ± S.D. of the P2X1 receptor density in control individuals and BPH patients normalized using as a reference protein the highly conserved GAPDH enzyme (EC 1.2.1.12, MW approx. 37 kDa). The number of individuals per group is shown below each bar. *$P < 0.05$ (unpaired Student's t-test corrected for multiple comparisons using the Holm-Sidak method) represent significant differences compared with control individuals.

The selective P2X1 antagonist NF023 applied in a concentration (3 μM) that was able to prevent ATP-induced contractions of the human detrusor, failed to affect ($P > 0.05$) nerve-evoked [³H]ACh release from detrusor strips isolated either from control individuals or from BPH patients (Fig. 5). Blockage of P2X3 and P2X2/3 receptors with A317491 (100 nM) and TNP-ATP (10 nM), respectively, lowered by a similar amount the nerve-evoked [³H]ACh release from control detrusor strips. The inhibitory effect of TNP-ATP (10 nM) was much more evident in detrusor samples from BPH patients (43% ± 6%, $n = 4$) than from control organ donors (27% ± 9%, $n = 4$), whereas the effect of A317491 (100 nM) was kept almost unaltered in the two

groups (24% ± 4%, $n = 5$ in BPH patients vs. 20% ± 2%, $n = 4$ in controls) (Fig. 5). It is, however, worth noting that A317491 (100 nM) and TNP-ATP (10 nM) were both inactive on ATP-induced human bladder contractions (see Fig. 2).

Discussion and Conclusions

In a previous study, we showed that cholinergic neurotransmission was increased in mucosal-denuded detrusor strips from BPH patients compared with control organ donors and that this could have been the result, at least in part, of deficits in the activity of ecto-NTPDase1/CD39 subsequently unbalancing extracellular
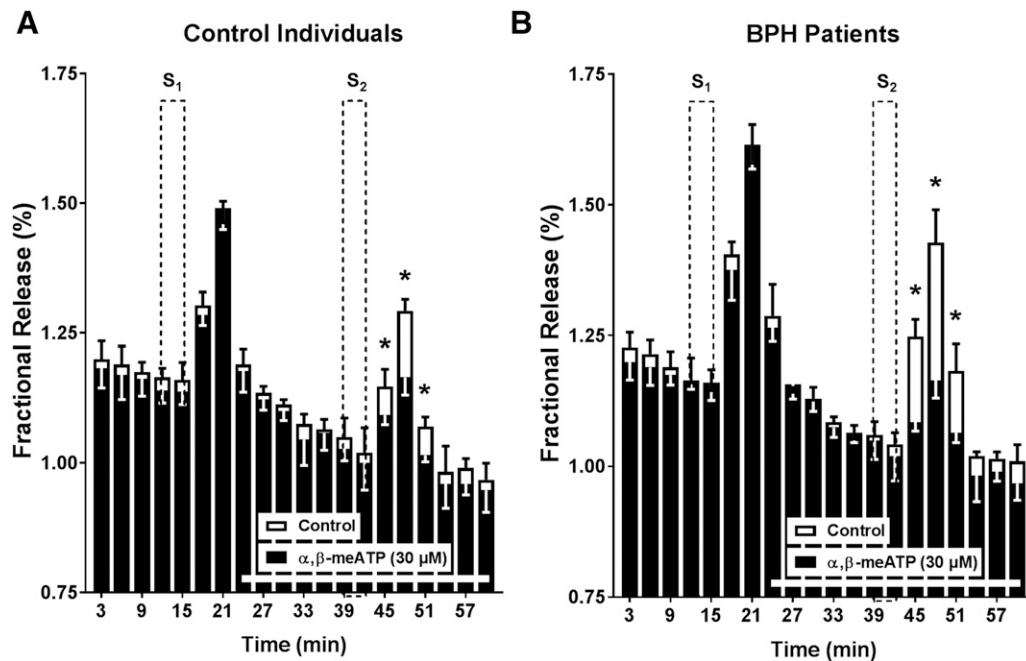
Fig. 4. Desensitization of ionotropic P2X1-3 receptors with α,β-MeATP reduced the release of $[^3H]$ACh from stimulated urothelium-denuded detrusor strips from (A) control individuals and (B) BPH patients. Tritium outflow (ordinates) is expressed as a percentage of the total radioactivity present in the tissue at the beginning of the collection period (Fractional Release, %). Abscissa indicates the times at which samples were collected. $[^3H]$ACh release was elicited by electrical field stimulation (10 Hz, 200 pulses of 0.5 milliseconds' duration) twice, starting at 12th (S_1) and 39th (S_2) minutes after the end of washout (zero time). α,β-MeATP (30 μM, black bars) was added to the incubation media 15 minutes before S_2 (horizontal white bar); for comparison, we show a time course of tritium outflow in control conditions (white back bars) where no drug was added during the collecting period. The vertical bars represent S.D. of four (A) to six (B) different individuals. Note that the spontaneous tritium outflow was not changed in the presence of α,β-MeATP (30 μM).*$P<0.05$ (unpaired Student's t-test corrected for multiple comparisons using the Holm-Sidak method) represent significant differences when compared to control individuals.

ATP accumulation and endogenous adenosine formation (Silva-Ramos et al., 2015). Here, we showed that extracellular ATP accumulation increases detrusor tension in obstructed patients by two synergistic mechanisms. One is mediated through direct activation of ionotropic P2X1 receptors, which are overexpressed on smooth muscle fibers of obstructed human bladders. The second is operated indirectly through facilitation of ACh release from cholinergic nerves via the activation of P2X2/3 receptor heterotrimers sensitive: 1) to PPADS, TNP-ATP, and A317491 antagonists and 2) to desensitization by the enzymatically-stable ATP analog α,β-MeATP. The unbalance between the excessive ATP-mediated P2X2/3 receptor facilitation of ACh release and the loss of the adenosine A_1 receptor-mediated inhibitory tone of cholinergic nerve terminals contributes to increased nerve-evoked detrusor reactivity in obstructed patients with BPH. Although the purinergic-related detrusor overactivity may be a burden for patients with BOO, which is certainly worth being explored therapeutically using P2X receptor antagonists and/or adenosine A_1 receptor modulators (e.g., receptor agonists, nucleoside uptake, and deaminase inhibitors), its maintenance/potentiation in later stages of the disease may be attractive to overcome detrusor underactivity. Even though we did not explore this possibility specifically, we could have increased ATP-mediated contractions of detrusor strips from BPH patients using a competitive inhibitor of human NTPDase1/CD39, ARL67156 (100 μM). Therefore, one can speculate that manipulation of the purinergic tone is even more relevant to overcoming DU, taking into consideration that cholinergic activity is substantially impaired in obstructed patients owing to BPH (see below).

The effect of BOO on ACh release from bladder nerves has only been studied in animal models. In a rat model of BOO, nerve-evoked ACh release (normalized by the wet tissue weight) decreased in parallel to the reduction in the density of bladder nerves (Murakami et al., 2008). Nevertheless, nerve density changes must take into consideration that obstructed bladders exhibit hypertrophy of the smooth muscle fibers and intercellular accumulation of collagen and elastic fibers causing a 4-fold increase in the bladder weight corrected by the total body weight. Therefore, if one corrects the amount of ACh release by the increase in bladder weight owing to BOO in the Murakami study, we easily reach the conclusion that the absolute amount of ACh release triggered by electrical stimulation is similar (or even higher) in obstructed than in control bladders, and the same certainly occurs with the number of nerves innervating the bladder of the two animal groups. More importantly, experimental evidence supports the idea that animal models of BOO do not exactly mimic the pathologic features observed in obstructed bladders of men with BPH. Whereas BOO causes very rapidly rigid bladders in animal models, the bladder of obstructed BPH patients is fairly flexible and rigidity is progressive and usually takes several years. The bladder mass of BPH patients increases slowly and hardly doubles the original weight (Kojima et al., 1996), which is far from the 4-fold increase in the bladder weight gain observed 2 weeks after surgery to partially occlude the bladder outlet in the rat (Murakami et al., 2008; see also Hampel et al., 2002). Rapid development of smooth muscle hypertrophy and interstitial fibrosis: 1) supports the inverse relationship between detrusor contractility and the
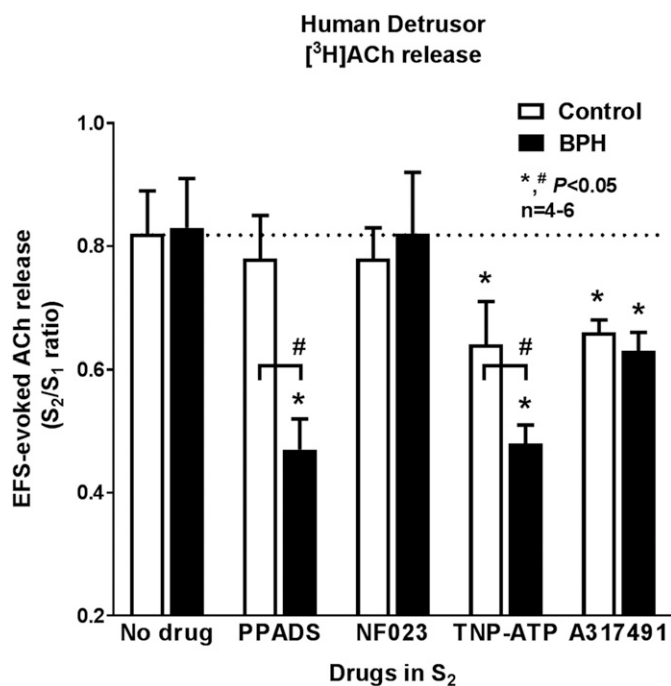
Human Detrusor
[³H]ACh release

Fig. 5. Tonic activation of P2X2/3 heterotrimers downregulates superfluous [³H]ACh release from cholinergic nerves innervating the detrusor of BPH patients. PPADS (10 μM, a nonselective P2 antagonist), NF023 (3 μM, a selective P2X1 antagonist), TNP-ATP (10 nM, a preferential P2X2/3 antagonist), and A317491 (100 nM, a selective P2X3 antagonist) were applied 15 minutes before S$_2$. The ordinates are S$_2$/S$_1$ ratios, i.e., the ratio between the amount of tritium collected during the second period of stimulation and that obtained during the first stimulation period when no drug was added; the S$_2$/S$_1$ ratios obtained in the absence of any drug are also shown from comparison (left-hand side bars). The data are means ± S.D. of four to six individuals. [*,#]$P < 0.05$ (two-way ANOVA followed by the Sidak's multicomparison test with a single pooled variance) represent significant differences compared with the situation in which no drug was added or with the effect of the same drug in control individuals, respectively.[*]$P < 0.05$ (two-way ANOVA followed by the Sidak's multicomparison test with a single pooled variance) represent significant differences when compared to the situation where no drug was added; [#]$P < 0.05$ (two-way ANOVA followed by the Sidak's multicomparison test with a single pooled variance) represent significant differences when compared to the effect of the same drug in control individuals.

degree of outlet obstruction in these animals (Moore et al., 2002; Austin et al., 2004) and 2) may explain why the detrusor decompensates so early in the course of experimental obstruction (Michel and Barendrecht, 2008), a situation that is observed only at later stages of the human disease.

Previous studies using obstructed human bladder samples admitted that this pathologic condition was associated with a partial bladder denervation compared with control specimens (Harrison et al., 1987; Gosling, 1997). Unfortunately, these authors did not directly assess cholinergic innervation of the human bladder and, more importantly, they also failed to actually measure bladder denervation, i.e., the reduction in the number of nerve fibers. As argued above regarding animal studies, a decrease in the density of nerve fibers within the bladder wall is not an index of bladder denervation, because one must take into account smooth muscle hypertrophy and more or less extensive interstitial deposition of extracellular matrix proteins (e.g., collagen, elastin), which moves apart nerve fibers and tiny axon terminals. The partial denervation hypothesis was sustained by functional studies showing that muscle biopsies from patients with detrusor instability owing

to BOO demonstrated supersensitivity to ACh and a reduction in nerve-mediated responses compared with stable bladders (Harrison et al., 1987). Yet, in this respect, functional studies are not consensual (Turner and Brading, 1997; Michel and Barendrecht, 2008). We are aware that discrepancies among detrusor responsiveness to ACh in BPH- versus control-patients may be a consequence of age differences between the two groups (Yoshida et al., 2001; Mansfield et al., 2005). Here, we excluded this possibility, because no significant correlation between age and the magnitude of ACh-induced detrusor contractions was observed.

Notwithstanding this dispute, we found that electrical field stimulation of urothelium-denuded detrusor strips from obstructed BPH patients releases higher amounts of [³H]ACh compared with controls. Thus, if the partial denervation hypothesis is confirmed, our data suggest that the amount of ACh released per nerve unit must be highly enhanced in the bladder of BPH patients. Interestingly, we also showed here that the amplitude of ACh-induced contractions was significantly reduced in obstructed BPH bladders compared with controls, a situation similar to that observed regarding carbachol-induced contractions in rats tested 6 months after surgery to cause BOO (Murakami et al., 2008). Reduction in cholinergic sensitivity of detrusor smooth muscle fibers would attenuate, rather than increase, nerve-evoked contractions of obstructed human bladders. To explain neurotransmission strengthening in the obstructed human detrusor, one must take into account reinforcement of the purinergic component leading: 1) to the release of excessive amounts of ACh from cholinergic nerve efferents, and/or 2) to the activation of overexpressed P2X1 receptors on detrusor smooth muscle fibers by released ATP.

Data from our study suggest that anticholinergic agents may be less effective in treating detrusor overactivity in BPH patients. Since the majority of these compounds are competitive muscarinic antagonists preferentially blocking M$_3$ receptors on smooth muscle fibers, their action may be substantially hampered in obstructed BPH patients because ACh release is facilitated, leading: 1) to superfluous levels of the transmitter in the synaptic cleft, and/or 2) to muscarinic receptor desensitization by excessive cholinergic exposure, as has been demonstrated in the rat BOO model (Braverman and Ruggieri, 2003). It thus appears that the net cholinergic neurotransmission in obstructed bladders from BPH patients is partially compensated by the increase in ACh release from cholinergic nerve terminals, but this does not fully explain detrusor overactivity often seen in BPH patients unless one hypothesizes the release/accumulation of a second extracellular transmitter, like ATP, being responsible for increasing the activity of cholinergic nerve terminals and/or the detrusor contraction. According to this theory, blockage of purinergic signals may be a valuable alterative for the treatment of detrusor overactivity in BPH patients resistant to antimuscarinics.

The role of ATP in the process of enhancing detrusor contraction in obstructed patients seems pivotal. ATP has long been considered a "danger-signaling molecule" in many organ systems and the nucleotide undertakes important roles in tissue remodeling secondary to injury (Burnstock and Verkhratsky, 2010). The exact mechanism by which the purinergic signaling cascade is activated in the detrusor of BPH patients is not yet established. There is a considerable bulk of evidence showing that BOO causes tissue ischemia (Azadzoi et al., 1996; Saito et al., 1997; Belenky et al., 2003;

Yamaguchi et al., 2014) and this may trigger the release of purines from affected cells. Experimentally-induced ischemia secondary to hypoxia-reoxygenation cycles increases atropine-resistant nerve-mediated contractions in the rat detrusor, an effect that can be blocked by P2X receptor antagonists (Elliott et al., 2013). Furthermore, ischemia lowers tissue pH, which is usually associated with deficits in the activity of NTPDases leading to extracellular ATP accumulation (Kukulski et al., 2005) along with the upregulation of ionotropic P2X receptor expression and activity (Zhang et al., 2014). Summation of acidic pH and painful purinergic signals have recently been demonstrated to occur via unilateral interaction between acid-sensing ion channels (e.g., ASIC3) and ATP-sensitive P2X3 receptor protein complexes through stimulation of the proton-binding site and downstream activation of Ca^{2+}-dependent pathways (Stephan et al., 2018), but the presence of such complexes has not been documented yet in the human bladder. Although we did not address these issues in this study, ischemia-induced purinergic signaling reinforcement may be a mechanism worth exploring in the future to explain the pathophysiology of detrusor overactivity in BPH patients.

This study showed for the first time that endogenously released ATP facilitates ACh outflow from urothelium-denuded human detrusor strips and this effect seems to be strengthened in obstructed bladders of BPH patients. Facilitation of ACh release by ATP has been previously shown in other synapses (Magalhães-Cardoso et al., 2003; Sperlágh et al., 2007). Our results suggest that ATP-mediated facilitation of ACh release is dependent on the activation of ionotropic P2X1-3 receptors, as these receptor subtypes are only amenable to desensitization by α,β-methylene ATP. Similar results were obtained in the porcine detrusor (D'Agostino et al., 2012). In contrast to the well known contractile effect of ATP in the human detrusor via activation of the P2X1 receptor, this receptor subtype failed to participate in ATP neuromodulation. This was concluded because the selective P2X1 receptor antagonist NF023 was devoid of effect on nerve-evoked ACh release from detrusor strips of control organ donors and BPH patients. Blockage of P2X2/3 heterotrimers with TNP-ATP, as well of the P2X3 receptor with its selective antagonist, A317491, significantly decreased the release of ACh from stimulated detrusor strips of control individuals. Interestingly, the inhibitory effect of TNP-ATP almost doubled its magnitude in samples collected from obstructed BPH patients, whereas the effect resulting from selectively blocking the P2X3 receptor with A317491 remained unaltered. These findings suggest that both P2X2/3 and P2X3 receptors may be involved in ATP-mediated facilitation of nerve-evoked ACh in the human bladder, but augmentation of the purinergic neuromodulatory tone in obstructed bladders of BPH patients may result from upregulation of the P2X2/3 receptor subtype. The presence of P2X3 receptors and P2X2/3 heterotrimers has been detected in peripheral nerves by immunofluorescence microscopy (Dunn et al., 2001). These receptors have been found in suburothelium nerve fibers of the human bladder (Brady et al., 2004) and their presence was also detected in nerve bundles of rat and human detrusors (Lee et al., 2000; Neuhaus et al., 2012).

In conclusion, data produced here and in previous studies support the idea that in obstructed bladders of BPH patients several mechanisms are activated toward the generation of stronger nerve-evoked detrusor contractions. These include: 1) deficits in extracellular ATP hydrolysis leading to 2) increased contractile responses mediated by overexpressed postjunctional P2X1 receptors, 3) activation of facilitatory P2X2/3 heterotrimers and decreased adenosine A1-receptor-mediated inhibitory tonus on cholinergic nerves that result in 4) facilitation of ACh release from stimulated nerve terminals, which is 5) partial compensation for the lack of sensitivity of cholinoceptors present on smooth muscle fibers. Recent publications have pointed out that P2X3 (and/or P2X2/3) antagonists could be potential candidates for the treatment of painful bladder and overactive bladder syndromes (Ford and Undem, 2013; Smith et al., 2013). Besides demonstrating the relevance of P2X3 receptors in mediating bladder sensations, our data add important information concerning their effect on cholinergic neuroexcitability and detrusor overactivity that must be also taken into consideration. Our results also shed light on possible ways to increase bladder performance in situations of detrusor underactivity resulting from BOO. At present there is no pharmacological treatment of detrusor underactivity, and this is a clear unmet medical need (Osman et al., 2014). It is an appealing prospect to enhance detrusor performance in these patients through the activation of presynaptic P2X2/3 receptors, either by using subtype-specific receptor agonists or by inhibiting the activity of NTPDase1/CD39, as we did with ARL67156.

Authorship Contributions

Participated in research design: Silva-Ramos, Silva, Faria, Ferreirinha, Correia-de-Sá.

Conducted experiments: Silva-Ramos, Silva, Faria, Ferreirinha, Correia-de-Sá.

Contributed new reagents or analytic tools: Correia-de-Sá.

Performed data analysis: Silva-Ramos, Silva, Faria, Ferreirinha, Correia-de-Sá.

Wrote or contributed to the writing of the manuscript: Silva-Ramos, Silva, Ferreirinha, Correia-de-Sá.

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Address correspondence to: Dr. P. Correia-de-Sá, Laboratório de Farmacologia e Neurobiologia/MedInUP, Instituto de Ciências Biomédicas de Abel Salazar (ICBAS) - Universidade do Porto (UP), R. Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal. E-mail: farmacol@icbas.up.pt