ISABEL SOFIA DIAS DA SILVA

TARGETING THE PURINOME TO CONTROL HYPERACTIVITY OF THE HUMAN URINARY BLADDER

Tese de Candidatura ao grau de Doutor em Ciências Biomédicas, submetida ao Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

Orientador – Professor Doutor Paulo Correia-de-Sá

Categoria – Professor Catedrático

Afiliação – Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

ISABEL SOFIA DIAS DA SILVA

TARGETING THE PURINOME TO CONTROL HYPERACTIVITY OF THE HUMAN URINARY BLADDER

Dissertation in fulfilment of the requirements for the PhD degree in Biomedical Sciences, submitted to Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

Supervisor – Professor Paulo Correia-de-Sá

Category – Full Professor

Affiliation – Instituto de Ciências Biomédicas Abel Salazar of the University of Porto

This research was partially supported by Fundação para a Ciência e a Tecnologia (FCT, FEDER projects: PTDC/SAU-OSM/104369/2008, REEQ/1168/SAU2005, REEQ/1264/SAU/2005, PEst-OE/SAU/UI215/2014 and UID/BIM/4308/2016), Associação Portuguesa de Urologia and by University of Porto / Caixa Geral de Depósitos (Investigação Científica na Pré-Graduação).

The author was in receipt of a PhD Studentship from FCT (POPH – QREN/FSE funding, SFRH/BD/88855/2012).

AGRADECIMENTOS

Começo por agradecer à Fundação da Ciência e Tecnologia (FCT) pela bolsa de doutoramento atribuída (SFRH/BD/88855/2012) sem a qual este doutoramento não teria sido realizado.

Agradeço aos responsáveis das instituições onde este doutoramento foi realizado, Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto em parceria com o Centro Hospitalar do Porto – Serviço de Urologia, por permitirem e facultarem as condições necessárias para a realização deste trabalho.

Agradeço ao meu orientador, Professor Doutor Paulo Correia de Sá, a quem considero ser o meu "Pai científico". Agradeço-lhe por toda a orientação, por assegurar as condições necessárias para a concretização deste trabalho, pelo conhecimento transmitido, pelos ensinamentos, pelo apoio, pela motivação e pelo exemplo de dedicação e paixão à ciência.

Agradeço a todos os colegas do laboratório de Farmacologia e Neurobiologia do ICBAS que participaram ativamente e de forma direta neste trabalho, são eles a Doutora Fátima Ferreirinha pela sua ajuda nos estudos de imagem e de biologia molecular, Dra. Teresa Magalhães Cardoso pela ajuda na quantificação das experiências de libertação de nucleósidos/nucleótidos e catabolismo por técnicas de HPLC, à Dra. Alexandrina Timóteo, Ana Filipa e à Sílvia Moreira que participaram na realização dos estudos urodinâmicos em ratazanas anestesiadas *in vivo*.

Agradeço a toda a equipa do Serviço de Urologia do Centro Hospitalar do Porto que colaborou comigo na recolha de tecido humano e recrutamento de doentes para colheita de urinas. Em particular o meu especial agradecimento para o Dr. Miguel Silva Ramos, Dr. LaFuente de Carvalho e Dr. Vitor Cavadas.

Agradeço à técnica do laboratório de urodinâmica, do Serviço de Urologia do CHP, Dra. Ana Lopes, pela amizade, pela colaboração na colheita de urinas em doentes de avaliação urodinâmica, pela ajuda na discussão de casos clínicos e interpretação de resultados das cistometrias. Pelos bons momentos partilhados!

Agradeço a todos os colegas do laboratório de Farmacologia e Neurobiologia do ICBAS pela amizade, apoio, carinho e motivação que ao longo destes anos contribuíram para o bom ambiente e harmonia do trabalho. São eles, Dra. Alexandrina Timóteo, Dra. Milaydis Sosa, Doutora Cátia Vieira, Prof. Graça Lobo, Prof. Adelina Costa, Prof. Laura Oliveira, Prof. Ana Patrícia Sousa, Prof. Mónica

Rodrigues, Doutora Fátima Ferreirinha, Dra. Teresa, Mestre Bruno Bragança, Doutor Bernardo Matos, Doutora Aurora Barbosa e Doutora Mariana Certal.

Agradeço ao Prof. Miguel Faria pela amizade, animação, otimismo e pelos bons momentos partilhados em conjunto aquando as noitadas, feriados e fins-de-semana de trabalho com tecido humano de dadores de órgãos.

Agradeço à Dra. Milaydis Sosa e à Doutora Fátima Ferreirinha por toda a amizade, apoio e ajuda na revisão linguística que realizaram ativamente nesta tese.

Agradeço o apoio, a amizade e o carinho das técnicas do laboratório de Farmacologia e Neurobiologia do ICBAS, D.Belmira Silva e D. Maria Costa e Silva.

Agradeço a amizade e o carinho de alguns amigos que estiveram ao longo destes anos a apoiar-me, são eles a Maria João Valente, Andreia Brandão e a Alexandra Campos.

Agradeço aos demais colegas do departamento de Imuno-Fisiologia e Neurobiologia e Imunogenética, por toda a amizade partilhada e encorajamento, são eles, Prof. Paula Ferreira, Encarnação Rebelo, Bárbara Leal e Elva Bonifácio.

Antes de terminar, não posso deixar de agradecer aos meus “pilares”, porto de abrigo, que é a minha família. Agradeço todo o apoio incondicional dos meus pais e irmão.

E por fim… o último mas sem dúvida o mais importante agradecimento vai para o meu namorado, Nuno. Agradeço todo o apoio e compreensão. Agradeço a paciência dos fins-de-semana que “perdemos” porque tive que ir fazer experiências, agradeço a sua companhia nesses fins-de-semana a simplesmente ver-me trabalhar. Agradeço por toda a força, confiança e coragem que me deu! Obrigada por estares sempre a meu lado!

TABLE OF CONTENTS

I. ABBREVIATIONS

[^{3}H]ACh, tritiated acetylcholine

AC, adenylyl cyclase

Acetyl-CoA, acetyl coenzyme-A

ACG, anterior cingulate gyrus

ACh, acetylcholine

AD, adrenaline

ADA, adenosine deaminase

ADP, adenosine diphosphate

AK, adenosine kinase

AMP, adenosine monophosphate

ATP, adenosine triphosphate

BK, large-conductance (Ca^{2+})-activated K^+ channel

HBP, hiperplasia benigna da próstata

BoNT, botulinum toxin

BOO, bladder outlet obstruction

BPH, benign prostatic hyperplasia

BPS, bladder pain syndrome

BR, bladder rat

BSA, bovine serum albumin

Ca^{2+}, calcium ion

cAMP, cyclic adenosine monophosphate

ChAT, choline acetyltransferase

CHO, chinese hamster ovary

CHP, Centro Hospitalar do Porto

CI, confidence interval

CNS, central nervous system

CoA, coenzyme A

CREB, cyclic AMP response element-binding protein

CTRL, control subjects

DAG, diacylglycerol

DIC, differential interference contrast

DWT, detrusor wall thickness

EDTA, ethylenediamine tetraacetic acid

EFS, electrical field stimulation

EGF, epidermal growth factor

EGFR, epidermal growth factor receptor

ENT, equilibrative nucleoside transporter

E-NTPDases, ecto-nucleoside triphosphate diphosphohydrolase

ERK, extracelular signal regulated kinase

EUS, external urethral sphincter

GAPDH, glyceraldehyde 3-phosphate dehydrogenase

G_i, inhibitory G protein

GPCRs, G-protein coupled receptors

G_s, stimulatory G protein

HBP, hiperplasia benigna da próstata

HPLC, high-performance liquid chromatography

HR, hazard ratio

HUT, higher urinary tract

HX, hypoxanthine

Hz, hertz

IB, immunoblotting

IC, interstitial cystitis

ICBAS, Instituto de Ciências Biomédicas Abel Salazar

ICC, interstitial cells of Cajal

ICS, international continence society

IHC, immunohistochemistry

INO, inosine

IP_3, inositol triphosphate

IUS, internal urethral sphincter

K^+, potassium ion

K_{ATP}, ATP sensitive potassium channels

kDa, kilodalton

LP, lamina propria

LUT, lower urinary tract

LUTS, lower urinary tract symptoms

MAPK, mitogen-activated protein kinase

MR, muscarinic receptors

ms, millisecond

MW, molecular weigh

NA, noradrenaline

Na^+, sodium ion

nAChR, nicotinic receptors

NANC, non-adrenergic non-cholinergic

NGF, nerve growth factor

NK1, neurokinin 1 receptor

NO, oxide nitric

NPPS, nucleotide pyrophosphatase / phosphodiesterases

NT5E, ecto-5'-nucleotidase

OAB, overactive bladder

OCTs, organic cation transporters

OCT1, organic cation transporter type 1

OCT3, organic cation transporter type 3

OD, overactive detrusor

PAC, pituitary adenylate cyclase

PAC1, pituitary adenylate cyclase-activating polypeptide type 1 receptor

PACAP, pituitary adenylate cyclase activating polypeptide

PAG, periaqueductal gray

PKA, protein kinase A

PKC, protein kinase C

PLC, phospholipase C

PMC, pontine micturition centre

PVDF, polyvenylidene difluoride

RT-PCR, reverse transcriptase polymerase chain reaction

S_1, first stimulus

S_2, second stimulus

SAH, s-adenosyl homocysteine

SCI, spinal cord injury

SDS, sodium dodecyl sulfate

SMA, smooth muscle actin

$T_{1/2}$, half degration time

TRP, transient receptor potential

TRPV1, transient potential vanilloid subtype 1 receptor

TRPV4, transient potential vanilloid subtype 4 receptor

TUI, trato urinário inferior

U, urothelium

UDP, uridine diphosphate

UTP, uridine triphosphate

UV, ultraviolet

V, volts

VDCAs, voltage-dependent anion channels

VIP, vasoactive intestinal peptide

VPAC2, vasoactive intestinal peptide receptor type 2

II. RESUMO

O trato urinário inferior é essencial para receber a urina produzida pelo rim, proceder ao seu armazenamento e permitir o esvaziamento voluntário da bexiga mediante reflexos complexos coordenados pelo sistema nervoso central através da informação veiculada pelos nervos periféricos aferentes e eferentes. A bexiga tem duas funções principais, (1) receber e acondicionar a urina, funcionando como um reservatório, e (2) esvaziar voluntariamente quando se encontra preenchida. Estas funções podem ser vistas como tarefas simples. No entanto, a regulação do funcionamento da bexiga é complexa levando a que as suas disfunções sejam muito comuns e com uma elevada repercussão na qualidade de vida dos doentes e das suas famílias, bem como um grande impacto económico na sociedade. Apesar dos esforços crescentes na investigação científica das disfunções urinárias, ainda existem muitas lacunas no conhecimento relativo aos mecanismos envolvidos nas doenças do trato urinário inferior (TUI). Estas falhas determinam a necessidade de mais estudos para satisfazer as necessidades clinicas associadas às patologias do TUI.

O conceito de “purinoma” foi recentemente introduzido na investigação científica e sabe-se, hoje, que a sinalização purinérgica tem enormes repercussões na fisiopatologia de diversas doenças, incluindo as disfunções do TUI. O “purinoma” engloba o conjunto de mecanismos que determinam a libertação celular de purinas (e/ou pirimidinas), a sua metabolização extracelular por enzimas específicas e os vários subtipos de recetores responsáveis pelas ações biológicas das purinas (e/ou pirimidinas) originais e dos seus metabolitos. Neste trabalho, estudou-se o envolvimento do sistema purinérgico e pirimidinérgico na fisiopatologia das disfunções da bexiga. Os resultados obtidos sugerem a existência de novos alvos terapêuticos relacionados com o “purinoma” como possíveis alternativas para o tratamento de doenças do TUI.

As experiências foram realizadas em tiras do corpo da bexiga humana provenientes de doentes com obstrução infravesical por hiperplasia benigna da próstata (HBP) e de dadores de órgãos sem patologia conhecida do TUI (grupo controlo). A seleção dos doentes e, respetivos, controlos, bem como os procedimentos de colheita das amostras de bexiga humana, foram realizados por médicos especialistas do Serviço de Urologia do Centro Hospitalar do Porto (CHP). Foram, ainda, realizados ensaios “*in vitro*” com fragmentos de bexiga isolados de

ratazanas Wistar Han, bem como experiências de urodinâmica *in vivo* com cateter intravesical em ratazanas anestesiadas. Todos os procedimentos foram previamente aprovados pela comissão de ética do CHP e do Instituto de Ciências Biomédicas Abel Salazar (ICBAS).

Os resultados obtidos nos ensaios *in vitro* mostram que a libertação urotelial de ATP normalizada pela acetilcolina (ACh) é cinco vezes maior no grupo de doentes obstruídos comparativamente com o grupo controlo. Estes resultados estão de acordo com a hipótese levantada pelo nosso grupo de investigação de que a medição dos níveis urinários de ATP pode ser considerada um biomarcador clínico de disfunção urotelial em doentes obstruídos (Silva-Ramos et al., 2016a).

Apesar das evidências de que o ATP é uma peça chave na fisiopatologia das disfunções do TUI, pouco se sabe sobre o papel dos nucleótidos de uridina encontrados na urina humana neste contexto. Experiências anteriores do nosso grupo de investigação demonstraram que a ativação de recetores do subtipo $P2Y_6$ sensíveis ao UDP aumentava a frequência miccional em ratazanas anestesiadas e que este efeito era mediado indiretamente pelo aumento da libertação de ATP pelo urotélio vesical (Carneiro et al., 2014) através de hemicanais contendo pannexina-1 (Timóteo et al., 2014). Não obstante, o papel dos recetores $P2Y_6$ nunca tinha sido explorado na bexiga humana. Os resultados obtidos neste estudo mostram que a ativação de recetores do subtipo $P2Y_6$ no urotélio humano promove a libertação não-neuronal de ATP (e ACh) tanto em doentes com obstrução infravesical como nos indivíduos controlo, embora os níveis de ATP detetados sejam significativamente maiores no grupo dos doentes portadores de HBP. Os dados experimentais mostraram ainda o envolvimento de hemicanais contendo pannexina-1 na libertação de ATP induzida por estimulação de recetores $P2Y_6$ no urotélio humano sugerindo a hipótese de que a manipulação destas vias pode constituir uma alternativa terapêutica eficaz para controlar a hiperatividade vesical em doentes obstruídos.

Previamente demonstrou-se que os doentes com obstrução infravesical por HBP exibem défices na conversão extracelular dos nucleótidos de adenina pelas E-NTPDases levando a uma acumulação exagerada de ATP no detrusor. Esta situação está normalmente associada à hiperatividade vesical por via da excessiva ativação de recetores ionotrópicos P2X sensíveis ao ATP localizados nos neurónios sensitivos suburoteliais (P2X2/3) e no detrusor (P2X1), mas também se pode dever indiretamente à redução dos níveis extracelulares de adenosina necessários à

ativação de recetores inibitórios do subtipo A_1 nos nervos colinérgicos (Silva-Ramos et al., 2015a). Resultados apresentados nesta tese revelam pela primeira vez a existência de recetores excitatórios dos subtipos P2X3 e/ou P2X2/3 sensíveis ao ATP nas terminações nervosas colinérgicas do detrusor humano. A ação destes recetores não é contrariada pela ativação de recetores inibitórios sensíveis ao ADP devido a uma deficiência no catabolismo extracelular de ATP nos doentes obstruídos portadores de HBP. A redução dos níveis endógenos de ADP promove a sobreexpressão de recetores inibitórios do subtipo $P2Y_{12}$, abrindo uma janela terapêutica aos agonistas destes recetores para o tratamento da hiperatividade colinérgica nos doentes com obstrução infravesical.

Desde 2013, os agonistas dos adrenocetores-β_3 (e.g. mirabegron) têm sido utilizados no tratamento dos sintomas da bexiga hiperativa em alternativa à perda de eficácia dos fármacos anti-colinérgicos. Apesar da sua aprovação e sucesso clinico existe alguma controvérsia relativamente ao mecanismo molecular subjacente ao efeito terapêutico desta classe de fármacos. O “ponto-chave” desta controvérsia reside no facto de se ter demonstrado que as concentrações plasmáticas atingidas com doses terapêuticas de mirabegron são insuficientes para causar relaxamento do detrusor humano *in vitro*. Recentemente, demonstrou-se que a ativação dos adrenocetores-β_3 com doses baixas de mirabegron inibia a libertação de ACh em preparações de bexiga humana *in vitro* (D’Agostino et al., 2015). Porém, não existem evidências inequívocas da presença destes recetores nos terminais nervosos colinérgicos do detrusor humano (ver e.g. Coelho et al., 2017; discutido em Okeke et al., 2017; Andersson, 2017; Griffin et al., 2017). Esta controvérsia é sugestiva de que o efeito inibitório do mirabegron sobre a libertação de ACh se pode dever a um mecanismo indireto mediado pela libertação retrógrada de um mensageiro originado a partir do AMP cíclico intracelular capaz de inibir a libertação do neurotransmissor, tal como a adenosina.

Os resultados mostram que a inibição da libertação de ACh causada por agonistas dos recetores β_3 é de facto dependente da libertação de adenosina por intermédio do transportador equilibrativo de nucleósidos, ENT1, localizado nas fibras musculares lisas do detrusor humano e da ratazana. Foi, ainda, possível demonstrar experimentalmente que a adenosina formada a partir do catabolismo intracelular do AMP cíclico gerado pela activação do recetor β_3 no detrusor atua como um mensageiro retrógrado por via da ativação de recetores pré-juncionais do subtipo A_1

e que estes são os responsáveis pela inibição da atividade colinérgica produzida pelos agonistas β_3. Apesar dos mecanismos moleculares responsáveis pela libertação de adenosina em resposta à estimulação dos recetores β_3 ainda necessitar de elucidação, foi possível provar o envolvimento da via da EPAC nessa sinalização sem que a proteína cinase A (PKA) tenha um papel significativo.

Os resultados mostram, ainda, que os doentes portadores de obstrução infravesical por HBP apresentam um aumento da densidade de recetores A_1 e β_3, e uma diminuição dos níveis de expressão do transportador equilibrativo de nucleósidos comparativamente com os indivíduos do grupo controlo. Apesar da limitação no transporte de adenosina para o meio extracelular, o controlo da libertação de ACh foi semelhante nos 2 grupos de indivíduos, porventura devido ao reforço da atividade β_3 pelo mirabegron e/ou da sobreexpressão dos recetores inibitórios A_1 localizados nas terminações nervosas colinérgicas.

Em conclusão, este trabalho permitiu elucidar mecanismos moleculares e propor novos alvos terapêuticos para o tratamento das disfunções do TUI, em particular da hiperatividade da bexiga secundária à obstrução infravesival por HBP. Neste contexto, foi possível concluir que a manipulação da atividade do purinoma, em várias das suas vertentes, pode contribuir para o controlo medicamentoso da bexiga hiperativa. Propomos o uso de (1) antagonistas seletivos dos recetores $P2Y_6$ no tratamento da disfunção urotelial em doentes com obstrução infravesical, uma vez que o bloqueio destes recetores diminui os níveis de ATP libertados; (2) antagonistas seletivos para os recetores P2X3 e/ou P2X2/3 e/ou agonistas seletivos dos recetores $P2Y_{12}$ para contrariar a excitabilidade nervosa colinérgica nos doentes obstruídos, e/ou (3) agonistas seletivos dos recetores A_1 em conjunto com o mirabegron, ou outros agonistas β_3, para aumentar a eficácia destes no tratamento da bexiga hiperativa. A utilização de uma abordagem molecular para aumentar o número e/ou função dos transportadores equilibrativos de nucleósidos (ENT1) na membrana plasmátiva dos doentes obstruídos poderá ser outra metodologia a ter em conta para melhorar a eficácia terapêutica dos agonistas β_3 nos síndromes de bexiga hiperativa.

III. ABSTRACT

The lower urinary tract (LUT) is essential to receive the urine produced by the kidney, to store it in the bladder and to allow voluntary emptying by complex reflexes coordinated by the central nervous system through the information conveyed by afferent and efferent peripheral nerves. The bladder has two main functions, (1) receiving and conditioning urine, functioning as a reservoir of urine, and (2) emptying voluntarily when it is full. These functions can be seen as simple tasks. However, bladder dysfunctions are very common and have a high impact on the quality of life of patients and their families; bladder dysfunctions are also a major economic burden for the society. Despite the increasing research efforts on this problem, there are still many gaps in our knowledge regarding the mechanisms involved in lower urinary tract symptoms (LUTS). These failures determine the requirement for further studies to meet the clinical needs inherent to LUTS.

The concept of "purinome" has recently been introduced in scientific research and it is known today that purinergic signaling has enormous repercussions on the pathophysiology of various clinical situations, including LUTS. This designation encompasses the set of mechanisms that determine the cellular release of purines (and/or pyrimidines), their extracellular metabolism by specific enzymes, and the various receptor subtypes responsible for the biological actions of both purines (and/or pyrimidines) and their metabolites. In this study, the involvement of the purinergic and pyrimidinergic system in the pathophysiology of bladder dysfunctions was studied. The results obtained highlighted the existence of new therapeutic targets related to the “purinome” as putative alternatives for the treatment of diseases of the LUT.

The experiments were performed on human bladder strips from patients with benign prostatic hyperplasia (BPH) and organ donors without known LUTS (control group). The selection of patients and their respective controls, as well as procedures for collecting human bladder samples, were performed by specialists from the Urology Service of the Porto Hospital Center (CHP). *In vitro* assays were also performed with bladder fragments isolated from Wistar Han rats. In addition, we perfomed *in vivo* urodynamic experiments after intravesical catheterism in anaesthetized rats. All procedures were previously approved by ethics committees of CHP and of the Abel Salazar Institute for Biomedical Sciences (ICBAS).

The results obtained in the *in vitro* assays show that the urothelial release of ATP normalized by acetylcholine (ACh) is five times greater in the group of obstructed patients compared to the control group. These results are in agreement with the hypothesis raised by our research group that the measurement of urinary ATP levels can be considered a clinical biomarker of urothelial dysfunction in obstructed human patients (Silva-Ramos et al., 2016a).

Despite the evidence that ATP plays a key role in the pathophysiology of LUT dysfunctions, little is known about the role of uracyl nucleotides found in human urine in this context. Previous experiments from our research group have shown that the activation of UDP-sensitive $P2Y_6$ receptor subtypes increased the voiding frequency in anaesthetized rats and that this effect was indirectly mediated by the increase in ATP release from the urothelium (Carneiro et al., 2014) through pannexin-1 containing hemichannels (Timóteo et al., 2014). Nevertheless, the role of $P2Y_6$ receptors had never been explored in the human bladder. The results obtained in this study show that activation of the $P2Y_6$ receptor subtype in the human urothelium favors the non-neuronal release of ATP (and ACh) both in obstructed patients and in control subjects. It is also worth noting that the $P2Y_6$ receptor-induced ATP release was significantly higher in the group of patients with BPH than in controls. Experimental data further suggest the involvement of pannexin-1 containing hemichannels in the release of ATP induced by stimulation of the $P2Y_6$ receptor in the human urothelium, thus raising the hypothesis that manipulation of these pathways may constitute an effective therapeutic alternative to control overactive bladder symptoms in obstructed patients.

Patients with infravesical BPH obstruction have previously been shown to exhibit deficits in the extracellular conversion of adenine nucleotides by E-NTPDases leading to surplus ATP accumulation in the detrusor. This situation is usually associated with bladder overactivity due to an excessive activation of ATP-sensitive P2X ionotropic receptors located in suburotelial (P2X2/3) sensory neurons and in the detrusor (P2X1). Nevertheless, it might also be due indirectly to the reduction of extracellular adenosine generated from the extracellular catabolism of ATP, which is is required to activate inhibitory A_1 receptors on cholinergic nerves terminals (Silva-Ramos et al., 2015a). Results presented in this thesis show for the first time the existence of excitatory receptors of the ATP-sensitive P2X3 and/or P2X2/3 subtypes on cholinergic nerve varicosities of the human detrusor, whose action is left

unrestrained by deficient activation of ADP-sensitive inhibitory receptors due to the impairment of extracellular ATP breakdown in obstructed patients with BPH. The reduction of endogenous levels of ADP favours overexpression of inhibitory receptors of the $P2Y_{12}$ subtype, opening a therapeutic window to agonists of this receptor subtype for the treatment of cholinergic hyperactivity in obstructed patients.

Since 2013, β_3-adrenoceptor agonists (like mirabegron) have been used in the treatment of overactive bladder symptoms as an alternative to the lack of efficacy of anti-cholinergic drugs. Despite the official approval and clinical success of these drugs there are still some controversies regarding the molecular mechanism underlying the therapeutic effects of this type of drugs. The major issue of this controversy is that the plasma concentrations achieved at therapeutic doses of mirabegron are not enough to cause relaxation of the human detrusor *in vitro*. Recently, activation of β_3 adrenoceptors with low doses of mirabegron has been shown to inhibit ACh release in human bladder preparations *in vitro* (D'Agostino et al., 2015). However, there is no clear evidence for the presence of these receptors on cholinergic nerve terminals of the human detrusor (see eg Coelho et al., 2017, discussed in Okeke et al., 2017; Andersson, 2017; Griffin et al., 2017) leading to the hypothesis that the inhibitory effect of mirabegron on ACh release may be due to an indirect mechanism mediated by the retrograde release of a messenger originating from intracellular cyclic AMP that is capable of inhibiting the release of the neurotransmitter, such as adenosine. The results show that inhibition of ACh release caused by β_3 receptor agonists is in fact dependent on adenosine release via the nucleoside equilibrating transporter, ENT1, located on smooth muscle fibers of the human and the rat detrusors. It was also possible to experimentally demonstrate that adenosine formed from the intracellular catabolism of cyclic AMP generated by activation of the β_3 receptor in the detrusor acts as a retrograde messenger through the activation of pre-junctional A_1 receptors, which are responsible for the inhibition of the cholinergic nerve activity caused by β_3 receptor agonists. Although the molecular mechanisms responsible for the release of adenosine in response to stimulation of β_3 receptors is still lacking, it was possible to prove an involvement of the EPAC pathway in this signaling pathway without much participation of the protein kinase A (PKA).

The results further suggest that A_1 and β_3 receptors are overexpressed in patients suffering from infravesical obstruction due to BPH, while the equilibrative

nucleoside transporter, ENT1, is downregulated, compared to control subjects. Despite these features leading to limitation of adenosine outflow to the extracellular medium, the control of ACh release was similar in both groups. This might be due to the relative increase in β_3 receptors activity by mirabegron and/or to overexpression of the A_1 inhibitory receptors located on the plasma membrane of cholinergic nerves of obstructed patients.

In conclusion, this work elucidated the underlying molecular mechanisms and proposed novel therapeutic targets for the treatment of LUTS, in particular of bladder hyperactivity secondary to infravesal obstruction due to BPH. In this context, one may suggest that manipulation of the “purinome”, in several of its aspects, can contribute to drug ability to control of overactive bladder syndromes. We propose the use of (1) selective $P2Y_6$ receptor antagonists for the treatment of urothelial dysfunction in patients with infravesical obstruction, since blockade of these receptors decrease the extracelular levels of ATP and, thus, its reactivity in the bladder; (2) selective antagonists of P2X3 and/or P2X2/3 receptors and/or selective agonists of the $P2Y_{12}$ receptor in order to counteract cholinergic hyperexcitability in obstructed patients, and/or (3) selective A_1 receptor agonists in conjunction with mirabegron, or other β_3 agonists, which may be necessary to increase the therapeutic efficacy of the latter in patients with overactive bladder. The use of a molecular approach to increase the number and/or activity of equilibrative nucleoside transporters (ENT1) in the plasma membrane of obstructed patients may be another strategy deserving attention to improve the therapeutic efficacy of β_3 agonists in overactive bladder syndromes.

IV. INTRODUCTION

1. Urinary tract

The urinary tract is composed by the kidneys, the ureters, the bladder and the urethra. The main function of this system is filtering the excess fluid and other substances from the bloodstream. In other words, it regulates the plasma composition through controlled secretion of salts, water and organic wastes (Vander et al., 2001; Provophys et al., 2006-2007). Urine is the final product of substances filtration from the blood, and is used to extract excess minerals or vitamins, as well as blood corpuscles from the body. The urinary system together with the other systems of the body contributes to maintain homeostasis (Provophys et al., 2006-2007; Drake et al., 2008).

The urinary tract can be subdivided into two groups: the higher urinary tract (HUT) and the lower urinary tract (LUT). The HUT is constituted by the kidneys and the ureters, while the LUT is composed by the bladder and the urethra (Vander et al., 2001; Provophys et al., 2006-2007; Drake et al., 2008).

1.1. LOWER URINARY TRACT

The LUT (Figure 1) has a complex physiology that includes both myogenic and neurologic aspects (Chapple, 2011). This system differs from other systems, because it dependents on the central nervous system (CNS) control, making a distinction from other structures that maintain a level of function even after the extrinsic neural input has been eliminated (Fowler et al., 2008).

1.1.1. Urinary bladder

The urinary bladder is a hollow organ with a muscular composition that allows it to be distensible or “elastic” during filling with urine. This organ is tetrahedral when empty and ovoid when filled (Macarak and Howard, 1999). As represented in Figure 1 the bladder can also be divided into two main components, the bladder body, which is located above the ureteral orifices, and the base consisting of the trigone, the

urethrovesical junction, the deep detrusor, and the anterior bladder wall (Andersson and Arner, 2004).

The bladder wall is formed by four layers of tissue, (1) the (innermost) mucosa (also denominated as urothelium), which separates the urine from the detrusor smooth muscle; (2) the submucosa; (3) the *muscularis*, a smooth muscle layer, also designated as detrusor muscle; and (4) the serosa, the continuation of the peritoneum (Van de Graaff and Ward Rhees, 2001). Towards the bladder base the ureters open into the lumen; the triangular region formed by orifices of both ureters and the bladder neck is called trigone (Figure 1) (Provophys et al., 2006-2007; Drake et al., 2008).

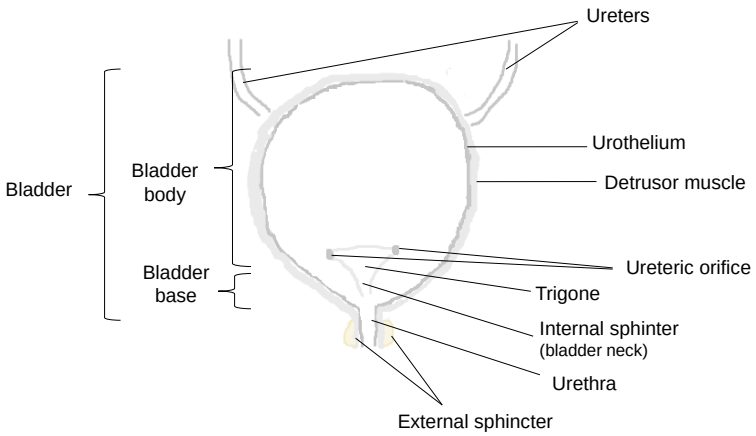


Figure 1. Parts of the lower urinary tract. The bladder is the main organ; it is lined by the urothelium and the detrusor smooth muscle to form a largest part of this organ.

The location of the urinary bladder in the human body depends on gender. In women the urinary bladder is located on the pelvic floor, while in men it is located on the superior pelvic floor due to the underlying prostate (Provophys et al., 2006-2007; Van de Graaff and Ward Rhees, 2001). The urinary bladder functions are urine storage and voiding. Urine storage of urine normally occurs under low filling pressure, which implies that the bladder relaxes during the filling phase. On the other hand, urine voiding requires a coordinated contraction of the bladder and relaxation of the urethra (Andersson and Arner, 2004).

Various types of cells are present in the bladder wall: smooth muscle cells, efferent nerve fibers, fibroblast-like cells and interstitial cells of Cajal (ICCs), also known as interstitial cells. Based on their localization within the bladder wall, several subgroups of ICCs have been identified (McCloskey, 2010; McCloskey, 2013). These

cells acts as an amplification stage in the sensory response to the bladder-wall stretch, as occurs during the bladder filling (Fry et al., 2007).

Both the detrusor and the mucosa are capable of independent spontaneous contractile activity (Kushida and Fry, 2016). If the two layers are in present the overall activity of the bladder is enhanced. Nevertheless, the contractile characteristics of both layers are not activated by similar stimuli, as judged by their responses to a wide spectrum of purinergic receptor agonists, capsaicin and extracellular pH changes (Kushida and Fry, 2016).

1.1.1.1. Urothelium

The urothelium, or uroepithelium, is a pseudostratified epithelium that covers the inner parts of the renal pelvis, the ureters, the urinary bladder, and the urethra. This epithelial cell layer grows on top of a basement membrane. It has a slow cellular turnover of approximately 6 months under normal circumstances (Walker, 1960; Lewis, 2000). Urothelial cells are specialized in the detection of both physical (e.g. distension, compression) and chemical (e.g. acids, inflammatory mediators) stimuli. The transducer role of the urothelium is enhanced by the close proximity of the urothelium to sensitive nerve afferents (Jen et al., 1995; Grol et al., 2008).

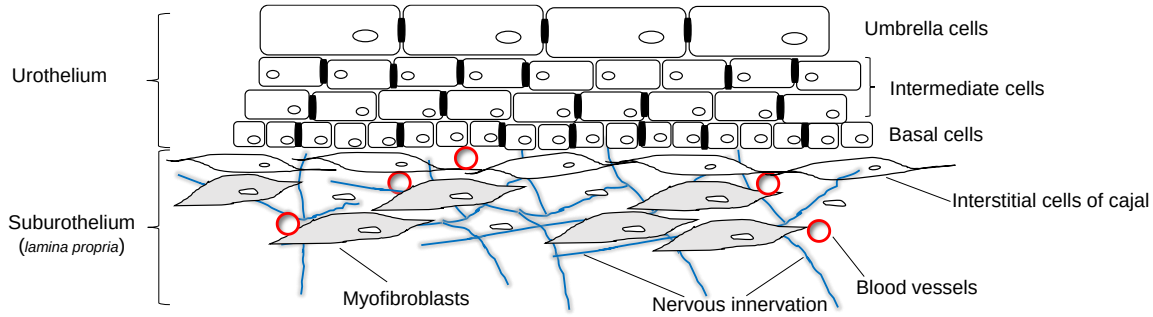


Figure 2. Scheme of the urothelium and suburothelium anatomy. The urothelium is composed of the umbrella, the intermediate, and the basal cells; while the suburothelium is constituted by the interstitial cells of Cajal, the myofibroblasts and the nerves.

The urothelium is formed by tree different cell layers, (1) a basal cell layer, (2) an intermediate cell layer and (3) an apical cell layer, which are represented in the Figure 2. The thickness of the urothelium reduces significantly during bladder filling mostly due to the fact that urothelial cells are highly deformable cells but also

because urothelial cell layers have the ability to run over each other to increase several fold the bladder capacity. This structural adaptation allows the urothelium to play several roles, (i) as a barrier to infections and damaging molecules, (ii) in the release of signaling molecules (signaling role), and (iii) in relaying sensory stimuli after detecting physiological and chemical stimuli (transducer role) (Birder and de Groat, 2007).

The cells of the innermost layer (apical layer) of the bladder urothelium are called umbrella cells and consist of large, flattened cells (Apodaca, 2004; Lewis, 2000). These cells are interconnected by tight junctions and are covered on their apical surface (nearly 70-80%) by crystalline proteins called uroplakins that assemble into hexagonal plaques (Hicks, 1975; Liang, 2001; Sun, 2006). The outermost layer of the urothelium consists of smaller basal cells that are separated from the suburothelial lamina propria (Birder, 2013). The small basal cells of the uroepithelium have properties typically seen in stem cells, such as, a slow turnover rate (Martin, 1972). Between umbrella and basal cells are located the intermediate cells (Figure 2) (Wu et al., 2009; Lewis, 2000; Jost et al., 1989). In some species, umbrella cells and perhaps also the intermediate cells, have projections to the basement membrane (Apodaca, 2004). The layer of intermediate cells may be different among species. In the urinary bladder of rodents there is one intermediate cell layer, whereas in humans there are up to five intermediate layers present (Walker, 1960; Lewis, 2000). The lamina propria layer is composed of several types of cells including fibroblasts, sensory nerve ending, myofibroblasts and ICCs (see Figure 2) (Birder, 2013).

During many years, it was believed that the urothelium had only one function: to act as a barrier against the bladder urine content. Increasing evidence demonstrate nowadays that the urothelium has many other roles which involve transport, regeneration, sensory functions, as well as the release of mediators (Guan et al., 2017). The urothelium is able to maintain high electrical resistance allowing only active ion transport between urine and blood (Lewis and Diamond, 1975). Urothelial cells exhibit “neuron-like” properties. Activation of urothelial cells by a variety of stimuli can trigger the release of various mediators (Table 1) which can impact on neural activity and, thereby, on bladder function (Birder, 2006). The mechanism underlying the release of these chemical mediators remains unclear and it is still not known whether different layers of the urothelium are responsible for secreting different mediators (Birder, 2011). On the other hand, there are a variety of

ion channels and receptors (see Table 1) which have been described throughout the urothelial layer from apical to basal cells (Guan et al., 2017; Wang et al., 2003; Lewis and Hanrahan, 1985). Afferent and efferent nerves are localized next to the urothelium, making the urothelium able to sense physical and chemical stimuli (Jen et al., 1995; Birder and de Groat, 2007).

Table 1. Signaling molecules and receptors expression in the urothelium.

MOLECULE	RECEPTOR	SPECIE	TECHNIQUE	REFERENCES
ACH	Muscarinic (M_1-M_5); Nicotinic ($\alpha_{3,5,7,9,10}$; β_3 e $_4$)	Rat; Human	IHC; IB; RT-PCR	Giglie et al., 2005; Tyagi et al., 2006; Kulman et al., 2008; Beckel et al., 2005; Bscheipfer et al., 2007.
ADENOSINE	Purinergic P1 (A_1, A_{2A}, A_{2B}, A_3)	Rat; Human	WB; RT-PCR; IH	Yu et al., 2006; Säve et al., 2009.
ATP	Purinergic P2 (P2X1-7 and $P2Y_{1,2,4,6}$)	Rabbit; Rat; Human	WB; IH; PCR; RT-PCR	Silva-Ramos et al., 2013a; Timóteo et al., 2014; Chopra et al., 2008; Tempest et al., 2014; Fergusson et al., 1997.
NA / AD	Adrenergic receptors (α_{1A}, $_{1D}$; β_{1-3})	Rat; Human	WB; RT-PCR	Ishihama et al., 2006; Michel and Vrgdar, 2006.
VIP/PACAP	PAC1, VPAC1, VPAC2	Rat	RT-PCR	Brass et al., 2005.
EGF	EGFR	Human	IHC	RØtterud et al., 2004.
SUBSTANCE P	Tachykinin (NK_1)	Human	RT-PCR/IB/IHC	Freiro et al., 2010.
BRADYKININ	Bradykinin (B2)	Rat	RT-PCR / IC	Chopra et al., 2005.
NO	-	Rat	Electrical recordings	Cho et al., 2017; Yoshimura et al., 2001; Ozawa et al., 1999; Birder et al., 1998.
NGF	-	Rat	Elisa	Suh et al., 2017; Yokokawa et al., 2016.
CYTOKINES	-	Dog	Fluorescence multiplex	Kang et al., 2013; Wood et al., 2012; Kruse et al., 2012.
PROSTAGLANDINS	-	Rabbit; Human	Radioimmunoassay; Elisa; functional studies	Downie and Karmazyn, 1984; Zhang and Bai, 2014.

Legend: ACH – acetylcholine; **NA** – noradrenaline; **AD** – adrenaline; **ATP** – adenosine triphosphate; **NO** – nitric oxide; **NGF** – nerve growth factor; **VIP** – vasoactive intestinal peptide; **PACAP** – pituitary adenylate cyclase activating polypeptide; **PAC** – pituitary adenylate cyclase; **PAC1** – pituitary adenylate cyclase-activating polypeptide type 1 receptor; **VIP** – vasoactive intestinal peptide; **VPAC1** – vasoactive intestinal peptide receptor type 1; **VPAC2** – vasoactive intestinal peptide receptor type 2; **NK_1** – neurokin-1 receptor; **EGF** – epidermal growth factor; **EGFR** – epidermal growth factor receptor; **RT-PCR** – reverse transcriptase polymerase chain reaction; **IHC** – immunohistochemistry; **IB** – immunoblotting.

Table 1 represents the signaling molecules and receptors detected in the urothelium which can be involved in urinary bladder functioning in health and disease: it is however, worth to note that the underlying mechanism(s) and role(s) of some of these molecules are still unclear (reviewed in Winter et al., 2014).

The urothelium can influences the contractile state of the detrusor smooth muscle through the release of mediators such as adenosine triphosphate (ATP) or nitric oxide (NO) (Santoso et al., 2010).

2. The micturition reflex

The urination or voiding, also known as micturition, is a process that begins in neonates as an involuntary process in which bladder emptying occurs without the conscious control of the individual (Beckel and Holstege, 2015). During voiding, the muscles of the urethra relax and the detrusor contracts, raising the intravesical pressure and causing the urine flow through the urethra. During urine storage the urethra is closed and the bladder smooth muscle is quiescent, allowing the bladder to distend and the intravesical pressure to remain low over a wide range of bladder volumes. The micturition reflex is coordinated by three sets of nerves (parasympathetic, sympathetic, and somatic) that emerge from the sacral and the thoracolumbar spinal cord (see Figure 3) (Fowler et al., 2008, Andersson, 1993; de Groat et al., 1993; Yoshimura and de Groat, 1997) involving pathways at many levels of the brain and multiple neurotransmitters (see Table 2) (Fowler et al., 2008).

The storage and elimination of urine depend on the coordinated activity of smooth and striated muscles in the two functional units of the lower urinary tract, namely the urinary bladder and the bladder outlet consisting of the bladder neck, the urethra and the urethral sphincter (Morrison et al., 2005; Fry et al., 2005). The complexity of this mechanism is demonstrated by the number of reflexes present in voiding. A total of 12 reflexes are involved in the urine release (Mahony et al., 1977).

Table 2. Innervation in the urinary bladder. Nerve structures, molecules (neurotransmitters), receptors, micturition phase and functions involved in the bladder function.

NERVOUS SYSTEM		NERVE STRUCTURES	MOLECULE	RECEPTORS	MICTURITION PHASE	FUNCTION
AUTONOMIC	Sympatic	Preganglionic motoneurons → hypogastric nerves	NA	α and β	Filling	Relaxation detrusor; Contraction internal sphincter
	Parasympatic	Motoneurons → pelvic nerve	ACh and ATP	Muscarinic and purinergic	Emptying	Contraction bladder; relaxation internal sphincter
SOMATIC		Motoneurons → pudendal nerve	ACh	Nicotinic	Filling and emptying	Input into the reflex circuits that control bladder filling and emptying

Legend: ACh – acetylcholine; **ATP** – adenosine triphosphate; **NA** – noradrenaline; **α** – adrenoceptor alpha subtype; **β** – adrenoceptor beta subtype.

The micturition reflex consists of two phases. Firstly, there is the filling phase, when urine is released into the bladder from the ureters; secondly, there is the emptying phase when urine is conveyed from the bladder to the urethra. During the filling phase, the bladder acts as a reservoir for the urine, maintaining low intravesical pressures. This phenomenon happens due to the absence of parasympathetic nerve activity and to the distensible properties of the bladder wall that allow the bladder to stretch to accommodate a continuously growing volume of urine (Yoshimura and de Groat, 1997). In this phase, mechano-sensitive receptors present in the urinary wall activate bladder afferent nerves, resulting in increased neural activity in the hypogastric and pelvic nerves (Sengupta and Gebhart, 1994; Floyd et al., 1976; de Groat and Lalley, 1972).

Activation of the sensitive system triggers a cascade of events: first-order afferent neurons convey sensory information, via pelvic nerves, to a cell group in the lateral dorsal horn and lateral part of the intermediate zone within the sacral spinal cord termed Gert's Nucleus (Holstege, 2005; Holstege, 2010).

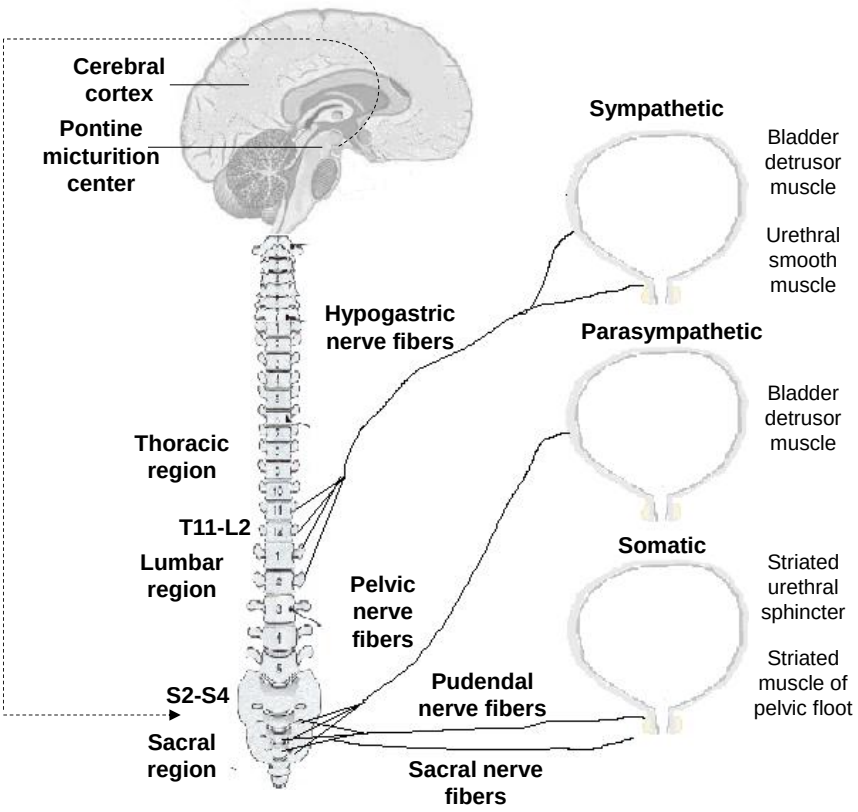


Figure 3. Micturition control. Coordination between the bladder and the outlet (bladder neck, urethra and urethral sphincters) is mediated by hypogastric nerve (sympathetic system), pelvic nerve (parasympathetic system) and pudendal nerves (somatic system). Adapted from Kanai and Andersson, 2010.

Together the complex interneuron circuitry within the spinal cord, parasympathetic innervation of the detrusor, is inhibited. The supraspinal input ensures that the voiding reflex remains under voluntary control as the decision to void is based on a combination of emotional, social and visceral sensation factors (Fowler et al., 2008). From the Gert's Nucleus, second-order afferent fibers ascend within the fasciculus gracilis to relay sensory information pertaining to bladder filling to the midbrain periaqueductal gray (PAG) matter (Griffiths and Tadic, 2008; Kavia et al., 2005), where third-order neurons originate. Higher centers such as the insula, the thalamus, the anterior cingulate gyrus (ACG), and the prefrontal cortices have multiple connections with the PAG. Bladder afferents received by PAG are relayed onto the insula-often referred to as the sensory cortex of the autonomic nervous system (Drake et al., 2010). In turn, the PAG acts as an interface between the afferent and the efferent limbs of bladder control circuits. The main centre of control of the micturition is located in the M-region or Barrington's nucleus, usually also

known as the pontine micturition centre (PMC). The PMC is located in the dorsal part of the caudal pontine tegmentum, adjacent to the locus coerulus (Fowler et al., 2008; Holstege, 2010). The PAG communicates with the PMC so that the higher centres ensure the maintenance of voluntary control of the voiding reflex. From the PMC, long fibres descend to the parasympathetic sacral bladder motoneurons and to the inhibitory interneurons to Onuf's nucleus (Holstege, 2010). When the bladder is full and a higher level of bladder distension is reached, the maximal bladder afferent activity within the PAG results in the stimulation of the PMC. As a result of this spinobulbospinal reflex, voiding occurs. The PMC is, therefore, regarded as the final efferent nucleus on the micturition pathways that co-ordinates inhibition of the sphincters and ignition of detrusor contraction. Hence, the activity of the PMC needs to be inhibited during the bladder's filling and storage phases (Shah et al., 2014).

Sacral parasympathetic (pelvic) nerves provide excitatory inputs (cholinergic (e.g. ACh) and purinergic (e.g. ATP)) to the bladder and have an inhibitory (nitrergic (e.g. NO) effect on the urethra (de Groat et al., 1993; Ralevic and Burnstock, 1998). Thoracolumbar sympathetic pathways release noradrenaline (NA) and provide excitatory inputs to the bladder neck and the urethra, as well as both facilitatory and inhibitory inputs to parasympathetic ganglia, and, in some species, have an inhibitory effect on the detrusor (Andersson, 1993; de Groat et al., 1993). These sympathetic pathways mainly run through sympathetic nerve trunks (Sugaya et al., 1988a). The lumbosacral efferent pathways in the pudendal nerves provide cholinergic excitatory inputs to the striated muscle of the urethral sphincter. Afferent activity in the bladder and the urethra is conveyed to the central nervous system by three sets of nerves (Figure 3 and Table 2) (de Groat et al., 1993; Yoshimura and de Groat, 1997; Morrison, 1999).

3. The cholinergic system

Acetylcholine (ACh) is a major regulator of the urinary bladder function, acting in the whole structural component of the bladder including the detrusor muscle, nerve terminals and the urothelium (Chess-Williams, 2002; Andersson and Wein, 2004; Birder, 2005; Hedge, 2006). The sources of ACh can be neuronal (Rang et al., 2004) or non-neuronal (Wessler et al., 2003). In the bladder, cholinergic neurons provide

neuronal ACh, while the non-neuronal ACh is mainly originated from urothelial cells (de Groat et al., 2004; Lips et al., 2007).

Table 3. Differences between neuronal and non-neuronal cholinergic system.

	NEURONAL	NON-NEURONAL
SYNTHESIS	Nerve terminal	Whole cell
STORAGE	Vesicles	Cytosol (?!)
RELEASE	Exocytosis synchronized demand	Transporter mediator continuously
RECEPTOR	Hot spots	Uniformly expressed
ACTION	Short lasting	Continuously
ELIMINATION	Rapid	Slow

ACh is synthetized by the action of choline acetyltransferase (ChAT), which catalyses the reaction between acetyl coenzyme-A (acetyl-CoA) and choline. ACh and CoA are the reaction products. Acetyl-CoA is originated in the mitochondria from the complete oxidation of pyruvate. The formation of acetyl-CoA is catalyzed by an enzyme complex called pyruvate dehydrogenase complex. Choline that is need for the synthesis of ACh comes from various sources, including the circulating choline in the plasma, the hydrolysis of phospholipids from cell membranes and from the uptake of choline resulting from action of acetylcholinesterase (AChE) on ACh released into the synaptic cleft. High affinity choline uptake is mediated by a concentrative transporter, which exhibits high-affinity for choline and it is dependent on temperature and the transmembrane Na^+ gradient. Synthesis of ACh is limited by the availability of choline (Rang et al., 2004). In neurons, synthesized ACh is transported into synaptic vesicles by an antiport mechanism. When an action potential reaches nerve terminals, it triggers Ca^{2+} influx from the extracellular milieu via voltage-sensitive channels, which promotes the fusion synaptic vesicles to the plasma membrane, and, consequently, the exocytosis of ACh to the synaptic cleft (Rang et al., 2004). Non-neuronal cells that produce ACh are not endowed with such effective vesicle storage. It is not even known whether the non-neuronal ACh is stored at all, but an equilibrium may exist between continuous synthesis, diffusion, release and hydrolysis (Wessler et al., 2003). The release mechanism of non-neuronal ACh is not known. However, ACh is an organic cation and it may be transported by ubiquitously expressed organic cation transporters (OCTs). This hypothesis was corroborated using direct inhibitors (quinine, corticosterone) or

substrate inhibitors (amiloride, verapamil) of OCTs which reduced ACh release (Wessler et al., 2003). The urothelium expresses two types of organic cation transporters, the type 1 (OCT1) and type 3 (OCT3) (Lips et al., 2007).

The ACh actions are conferred by two receptor families, G-protein-coupled muscarinic receptors (mAChR) and ionotropic nicotinic receptors (nAChR).

3.1. MUSCARINIC RECEPTORS

Muscarinic ACh receptors are responsible for bladder contractility (Andersson, 1993). These receptors belong to the family of G-protein coupled receptors (GPCRs), which are the most abundant and pharmacologically targeted plasma membrane receptors (Lander et al., 2001; Fredriksson et al., 2003). A common structural feature of GPCRs are the extracellular N-terminus, seven membrane spanning domains, three extracellular and three intracellular loops and an intracellular C-terminus. Stimulation of various GPCRs leads to activation of particular G-proteins and their intracellular signaling pathways, which control many important physiological functions. The intracellular signaling pathways of GPCRs involve the activation an effector enzyme to generate an intracellular second messenger (e.g. cyclic adenosine monophosphate (cAMP), diacylglycerol (DAG)) (Lodish et al., 2000). In addition to these well-established pathways, it has also been demonstrated that receptors also transduce non-G-protein-mediated signaling via arrestins and G-protein receptor kinases (Reiter and Lefkowitz, 2006; Lefkowitz and Shenoy, 2005; Lefkowitz, 1998).

Five subtypes of muscarinic receptors denoted as M_1-M_5 and encoded by five different genes have been discovered and characterized pharmacologically (Bonner 1989a, b; Bonner et al., 1987, 1988; Peralta et al., 1987; Kubo et al., 1986a). The M_1, M_3 and M_5 receptor subtypes activate preferentially $G_{q/11}$ guanine nucleotide binding proteins. These receptors alter cellular activity by stimulating phospholipase C (PLC) and generating the second messenger inositol triphosphate (IP_3) which induces the release of calcium from intracellular stores and DAG that causes activation of protein kinase C (Cauldfield and Birdsall, 1998; Jones et al., 1991). On the other hand, M_2 and M_4 receptors couple to $G_{i/o}$ proteins leading to inhibition of adenylate cyclase

(Peralta et al., 1988; Jones et al., 1991), decreases in cAMP levels and smooth muscle relaxation (Bolton and Zholos, 1997).

Table 4. Characterization of muscarinic receptors in the bladder.

G-PROTEIN COUPLED	INTRACELLULAR EFFECTS	MUSCARINIC RECEPTOR SUBTYPE	EXPRESSION IN THE BLADDER	FUNCTIONAL EVIDENCES
$G_{q/11}$	↑PLC → IP_3 → DAG	M_1	Urothelium; detrusor	No functional evidences
		M_3	Urothelium; detrusor; ICCs; myofibroblasts	Detrusor contraction
		M_5	Whole bladder?? But not in urothelium and detrusor isolated!!	No functional evidences
$G_{i/o}$	↓AC → ↓cAMP	M_2	Urothelium; detrusor; ICCs; myofibroblasts	Detrusor contraction
		M_4	Urothelium; detrusor	No functional evidences

Legend: PLC – phospholipase C; **IP_3** – inositol triphosphate; **DAG** – diacylglycerol; **AC** – adenylyl cyclase; **cAMP** – cyclic AMP; **M_1** – muscarinic receptor type 1; **M_2** – muscarinic receptor type 2; **M_3** – muscarinic receptor type 3; **M_4** – muscarinic receptor type 4; **M_5** – muscarinic receptor type 5; **ICC** – interstitial cells of Cajal.

Just like the majority of smooth muscle cells in many species, the smooth muscle of the urinary bladder, the detrusor, expresses a heterogeneous population of muscarinic receptors (see Table 4). With the exception of the muscarinic M_5 receptors, the other four subtypes of muscarinic receptors (M_1-M_4) were found in the uroepithelium and in the smooth muscle from human bladder (see Table 4; Tyagi et al., 2006). Notwithstanding this, the most relevant muscarinic receptors in urologic disorders seem to be of the M_2 and M_3 subtypes. The proportion of muscarinic M_2 and M_3 receptors found in the detrusor muscle of humans, guinea pigs and rabbits vary between 70-80% and 30-20%, respectively (Mansfield et al., 2005; Chess-Williams et al., 2001; Yamanishi et al., 2000). Despite the fact that the muscarinic M_3

density is lower than muscarinic M_2 receptors in the bladder of animals models (Yamanishi et al., 2000; Sellers et al., 2000) and humans (Yamanishi et al., 2015), the muscarinic M_3 receptor is the main responsible for detrusor smooth muscle contraction (Yamanishi et al., 2015; Yamanishi et al., 2000; Sellers et al., 2000).

Studies using M_3 knock-out mice (Matsui et al., 2000) showed that these animals have severe deficits in smooth muscle function, with bladder contraction to the muscarinic agonist carbachol being reduced by about 95% (Matsui et al., 2000). The remaining response was antagonized by the selective muscarinic M_2 antagonist, methoctramine, indicating a small M_2 receptor component. Furthermore, severe urinary retention was observed in male M_3 knock-out mice. These data suggest that smooth muscle contraction is predominantly mediated via muscarinic M_3 receptors and that muscarinic M_2 receptors play a minor role (Uchiyama et al., 2004). Experiments using M_2 knock-out mice confirmed the assumption that the muscarinic M_3 receptor is the predominant muscarinic receptor responsible for bladder contraction (Igawa et al., 2005). Interestingly, it was found that the muscarinic M_2 receptor subtype might play an important role in the local cholinergic modulation of the bladder afferent activity which contributes to bladder overactivity in healthy rats (Matsumoto et al., 2010).

The pharmacological characteristics of muscarinic receptors in the urothelium and in the detrusor smooth muscle are very similar (Ogoda et al., 2016), yet the specific role of these receptors in the urothelium is still unclear. Imaging studies demonstrated that immunoreactivity against muscarinic M_2 and M_3 receptors was greater in the urothelium of rats with bladder outlet obstruction (BOO) than in their control littermates, but only the muscarinic M_3 receptor was increased in the muscle layer of BOO animals (Kim et al., 2007). These results are compatible with the increased sensitivity to muscarinic agonists of the detrusor muscle of BOO animals (Speakman et al., 1987). The muscarinic M_2 receptor is also overexpressed rats exhibiting detrusor overactivity after spinal cord injury (SCI) (Matsumoto et al., 2012). These evidences demonstrated that muscarinic receptors are implicated in the pathophysiology of bladder disorders (Mukerji et al., 2006; Wu et al., 2014) and, thus, changes in these receptors deserve to be elucidated.

3.2. NICOTINIC RECEPTORS

The nicotinic receptors are heteromeric or homomeric cation channels, which exist as pentameric complexes of various combinations of α- and β-subunits (Sargent, 1993; Le Novére and Changeus, 1995; Lukas, 1995). The subtype composition determines essential channel characteristics such as ion preference, desensitization kinetics, and ligand specificity (Lukas et al., 1999; Elgoyhen et al., 2001). In the mammalian nervous system eight ligand binding α (α_2-α_7, α_9-α_{10}) and three structural β (β_2-β_4) subunits were identified (Mao et al., 2006). Different subunit combinations result in specific nicotinic receptors subtypes, with α_7 and α_9 being the only subunits capable of forming functional homomeric receptors (Mao et al., 2006).

In the bladder, activation of nicotinic receptors (i) facilitates the firing of mechano-sensitive afferents with terminals located near the bladder lumen, (ii) inhibits the firing of mechanosensitive afferents located near the serosal surface or in the muscle layers of the bladder, and (iii) excites unidentified-afferents (Yu et al., 2016). Neuronal nicotinic acetylcholine receptors play a key role in the control of bladder function mediating fast synaptic transmission between preganglionic and post ganglionic bladder efferents. Wherever it has been examined autonomic ganglia express more than one nicotinic receptor subtype (Poth et al., 1997; Mandelzys et al., 1994; Listerud et al., 1991) and bladder ganglia likely express multiple nicotinic receptor subtypes (De Biasi et al., 2000). The nicotinic receptors containing the α_3 and β_4 subunits are important for normal bladder function (Xu et al., 1999a,b). Neuronal nicotinic receptors expressed in pelvic neurons may contain combinations of $\alpha_3\beta_4$, $\alpha_3\beta_2$ and $\alpha_3\beta_2\beta_4$ subunits (De Biase et al., 2000). In the human bladder, α_7-homopentamers and α_9/α_{10}-heteropentamers (Beckel et al., 2006; Bschleipfer et al., 2007) were also described. These are preferentially permeable to calcium, and in culture cells from rat epithelium showed a rise in intracellular Ca^{2+} in response to nicotine (Beckel et al., 2006).

Nicotinic receptors present in the human bladder have an important role in the micturition control. It was also demonstrated that these receptors are involved in ATP release from bladder. Stimulation of different nicotinic receptors in the urothelium modulated ATP release in a reciprocal manner (Jenes et al., 2012; Beckel and Birder, 2012) with α_7 stimulation inhibiting ATP release and α_3 stimulation decreasing or increasing basal ATP release (Beckel and Birder, 2012).

4. The purinome

The concept of purinome is recent, and comprise the protein machinery involved in the biological interplay of extracellular purine and pyrimidine ligands, with the purinergic receptors, enzymes, transporters/channels (Volonté and D'Ambrosi, 2009). The novelty in the definition of purinome consists in grouping together known elements (ligand, receptors, transporters/channels and degrading enzymes) that contribute as a whole and not singularly, to the triggering maintenance and termination of purinergic signaling (Volonté and D'Ambrosi, 2009).

In the humans, is described that approximately 3266 proteins use purines as cofactors. The human purinome encompass a wide-ranging functional repertoire and many of these proteins are attractive drug targets (Murray and Bussiere, 2009). On the other hand, purinergic signaling is not only regulated by the presence of ligands together with receptors, enzymes and transporters, but also by their broad-spectrum of direct/indirect interactions with a previously unrecognized cooperative network, defined as purinome (Volonté and D'Ambrosi, 2009).

4.1. THE PURINERGIC SYSTEM

Purinergic signaling is a primitive system (Burnstock, 1996) that is involved in many non-neuronal and neuronal mechanisms, in both short-term and long-term events (Abbracchio and Burnstock, 1998; Burnstock and Knight, 2004). These include exocrine and endocrine secretion, immune responses, inflammation, mechanosensory transduction, platelet aggregation and endothelial-mediated vasodilatation, and in cell proliferation, differentiation, migration and death in development and regeneration, respectively (Burnstock, 2006).

The parasympathetic purinergic nerve-mediated component of contraction of the human bladder is small under normal situations, but it increases up to 40% in pathological conditions, such as interstitial cystitis (IC), outflow obstruction, idiopathic instability and some types of neurogenic bladder (Burnstock, 2001). These findings support the implications of the purinergic system in the pathophysiology of urinary bladder dysfunctions.

4.2. THE ROLE OF ATP

Adenosine triphosphate (ATP) was discovery in 1970 as the transmitter responsible for the non-adrenergic non-cholinergic (NANC) neurotransmission in the gut and the urinary bladder (Burnstock et al., 1972). ATP is a multifunctional ubiquitous biological molecule that acts as the primary intracellular energy source for all living cells and also as an extracellular signaling molecule. In the urinary bladder, ATP is the main signaling molecule with a pivotal role in bladder fullness sensation and in various bladder disorders (Burnstock, 2003).

Urothelium is the main source of ATP in the urinary bladder. Notwithstanding this, the extracellular amount of ATP significantly depends on the urothelium site, basal vs apical, as it largely depends on the expression and/or activity of co-localized ecto-nucleotidases (see below, section 4.4, Ecto-NTPDases). The larger purinergic tone may be partially related to the impairment of ecto-NTPDase1/CD39 activity, thus limiting the extracellular breakdown of ATP (Silva-Ramos et al., 2016b). Bidirectional release of ATP from *in vitro* rabbit urothelium demonstrated that stretching the tissue resulted in a transient fivefold increase in the rate of mucosal ATP release and a transient sixfold increase in serosal ATP release (Lewis and Lewis, 2006). These findings led to the conclusion that ATP may act as an important autocrine (Wang et al., 2005) and paracrine mediator (Cockayne et al., 2000) in the urinary bladder.

The filling of the urinary bladder stretches the urothelium and thereby activates mechanotransduction pathways, which are likely initiated by increased tension at the apical surface of the umbrella cells. These external mechanical stimuli induce stretch-activated ATP release from urothelial cells (Fergusson et al., 1997; Cheng et al., 2011; Dunning-Davies et al., 2013). ATP starts the purinergic mechanosensory transduction mechanism by acting on P2X3 homomultimeric and P2X2/3 heteromultimeric receptors on subepithelial sensory nerves, thus sending impulses to initiate the micturition reflex (Cockayne et al., 2005; Burnstock, 1999). Sensory information from the urinary bladder is conveyed by both lumbar splanchnic and sacral pelvis nerves to the spinal cord. Mechanosensitive properties of single afferent fibers in these two pathways showed that both low and high threshold stretch-sensitive afferents are present in both pathways (Xu and Gebhart, 2008). Single unit analysis of sensory fibers in the mouse urinary bladder revealed both low-and high

threshold fibers sensitive to ATP contributing to physiological (non-nociceptive) and nociceptive mechanosensory transduction, respectively (Rong et al., 2002).

Although ACh acting on smooth muscle muscarinic receptors is generally accepted as the primary effector controlling the bladder emptying, neural stimulation of the bladder is only partially inhibited by atropine, the anticholinergic drug (non-selective muscarinic antagonist) (Cowan and Daniel, 1983). This fact may be explained by the dual effect of ATP in the bladder. Although this nucleotide is involved in bladder filling sensation of the micturition reflex, it may facilitate voiding of urine from the bladder. ATP is co-release with ACh from parasympathetic nerves supplying the bladder. ATP release from cholinergic nerves exerts its activity by activating ionotropic P2X1 receptors which prevails in the detrusor smooth muscle in order to favor contraction of the bladder (Kennedy et al., 2007). The molecular mechanisms underlying ATP effects under pathological conditions are partially unknown and deserve to be explore given that excessive purinergic signals are detected in overactive bladder syndromes. Notwithstanding, in patients with overactive detrusor (OD) (Chen et al., 2010) and overactive bladder (OAB) (Chen et al., 2013), ATP reveals to be an important factor for initial perception of need to voiding, indicated by first desire to void (Chen et al., 2013). These support the evidences that the ATP may mediate initial afferent sensation in patients with dysfunctions characterized by urgency (Chen et al., 2013). On the other hand, ATP concentrations remained unchanged after voiding, suggesting that voiding did not further induce ATP release into intraluminal fluid (Chen et al., 2014). However, in patients with OAB (Silva-Ramos et al., 2013) and bladder outlet obstruction (BOO) (Silva-Ramos et al., 2016a), the urinary levels of ATP were increased, similar was also observed in patients with neurogenic bladder syndromes (Munoz et al., 2012). These reports sustain the hypothesis that ATP may a urinary biomarker to lower urinary tract dysfunctions (Silva-Ramos et al., 2013; Silva-Ramos et al., 2016a; Cheng et al., 2010; Munoz et al., 2011).

4.2.1. Mechanisms of ATP release

The mechanisms that are involved in the release of ATP from the urinary bladder are still a matter of debate in the literature. Besides ATP exocytosis from bladder nerves (Novak, 2003), there are several additional mechanisms putatively

involved in ATP release from non-neuronal cells (e.g. urothelial cells); these include efflux via connexin or pannexin hemichannels (Shout et al., 2002; Timóteo et al., 2014; Negoro et al., 2013), maxi-ion channels, ATP-binding cassette transporters, and P2X7 receptor channels (Burnstock, 2007).

As described above (section 4.2) ATP can be released both to the serosal and mucosal sites of the urothelium (Lewis and Lewis, 2006; Fergusson et al., 1997). ATP release from apical and basolateral membranes of the epithelial cells may involve the following mechanisms: (i) ATP-permeable release channels that may be present in each membrane domain and that are likely anion-permeable channels; (ii) plasma membrane forms of the mitochondrial porins or voltage-dependent anion channels (VDCAs); (iii) adenine nucleotide transporters; (iv) adenine nucleotide/nucleoside exchangers that are located in the mitochondrial membrane and may also be expressed in the plasma membrane and the ATP-filled vesicles (Schwiebert and Zsembery, 2003); (v) transient receptor potential (TRP) vanilloid receptor subtype 1 (TRPV1, Charrua et al., 2009) or TRPV4 channels (Mochizuki et al., 2009); (vi) ABC transporters (Wang et al., 2005) and (vii) mechanosensitive channels (Dunning-Davies et al., 2013; Birder et al., 2003).

4.3. PURINERGIC RECEPTORS

Purinergic signals in the urinary bladder are complex and depend on the activation of specific purinoceptor subtypes. These belong to two main families, identified as P1 and P2 for adenosine and adenine and/or uracil nucleotides, respectively (Burnstock, 1978). P2 purinoceptors are subdivided in ionotropic ligand-gated P2X channels and metabotropic G-protein-coupled P2Y receptors (Abbracchio and Burnstock, 1994). Regarding the P1 receptors four G-protein-coupled receptor subtypes (A_1, A_{2A}, A_{2B}, and A_3) have been cloned and pharmacologically characterized (Fredholm et al., 2001).

In the urinary bladder several cell types participate in the purinergic signaling cascade controlling bladder activity. These include urothelial cells (Schwiebert et al., 2003; Dunning-Davies et al., 2013), ICCs (Li et al., 2013), smooth muscle fibers (Yu et al., 2006; Gopalakrishnan et al., 2002) and suburothelial nerve fibers (Birder and Andersson, 2013; Gonzalez et al., 2014).

4.3.1. P2X receptors

P2X receptors are ATP-gated ion channels which mediate rapid (within 10 ms) and selective permeability to cations (Na^+, K^+ and Ca^{2+}) (North, 2002). They are distributed essentially on excitable cells (smooth muscle cells, neurons and glial cells) and their role is to mediate fast excitatory neurotransmission in response to ATP in both the central and the peripheral nervous systems. This contrasts with the slower onset of response (less than 100 ms) to ATP acting at metabotropic P2Y receptors.

The first cDNA encoding a P2X receptor (P2X1) was isolated in 1994 (Brake et al., 1994; Valera et al., 1994) and since then, seven different P2X receptor subunits have been identified (P2X1-7) (Abbracchio et al., 2009; Surprenant and North, 2009; North, 2002; Ravelic and Burnstock, 1998). The members of this receptor family show conserved subunit topology. They comprise two transmembrane spanning regions and possess intracellular amino- and carboxy-terminus which contain consensus binding motifs for protein kinases. A large extracellular loop exits which contains 10 conserved cysteine residues and is capable of forming a series of disulfide bridges. The first transmembrane is involved in channel gating while the second lines the ion pore in the membrane (North, 2002; Roberts et al., 2006). P2X receptor subunits are capable of forming functional homomeric and heteromeric trimers (North, 2002; Roberts et al., 2006).

Despite the presence of all types of P2X receptors in the urinary bladder (see Table 5), only the P2X1, P2X2, and P2X3 homomers and the P2X2/P2X3 heteromer have been primarily associated with bladder function (Ford et al., 2006; Cockayne et al., 2005). In general terms, the P2X1 receptor has been associated with the detrusor motor response (O'Reilly et al., 2001; Hoyle et al., 1989; Husted et al., 1983; Palea et al., 1994), whereas P2X2 and P2X3 subtypes are linked to bladder sensation (Smith, 2011). Both P2X1 and P2X3 receptors close quickly (hundreds of milliseconds) in the continued presence of ATP, whereas the P2X2 receptor remains open for the duration of ATP binding (Burnstock, 2000).

Table 5. Localization of P2X receptor subtypes in the urinary bladder.

P2X RECEPTOR SUBTYPES	LOCALIZATION IN THE BLADDER	SPECIES	REFERENCES
P2X1	Detrusor	Rat; human	O'Reilly et al., 2001; Lee et al., 2000; Carneiro et al., 2014; Silva-Ramos et al., 2015b; Moore et al., 2001; O'Reilly et al., 2002; Elneil et al., 2001.
P2X2	Detrusor intracellular; suburothelium; urothelium	Rat; human	Lee et al., 2000; Tempest et al., 2004; Svennersten et al., 2015.
P2X3	Detrusor; suburothelial nerves; urothelium	Rat; human	Lee et al., 2000; Vlaskovska et al., 2001; Elneil et al., 2001; Carneiro et al., 2014; Svennersten et al., 2015.
P2X4	Detrusor; nerve varicosities; capillary membranes	Rat; human	Lee et al., 2000; Moore et al., 2001; O'Reilly et al., 2002.
P2X5	Detrusor; epithelial cells	Rat	Lee et al., 2000.
P2X6	Detrusor; urothelial nucleic	Rat	Lee et al., 2000.
P2X7	Detrusor; urothelium	Human	Svennersten et al., 2015.

Due to the role of P2X2 and P2X3 receptors in bladder sensation and pain, various studies have been developed to understand their function in bladder diseases. In a systematic study of purinergic mechanosensory transduction in the mouse urinary bladder, it was shown that the activity initiated in pelvic sensory nerves through ATP release from the urothelium was mimicked by α,β-methylene ATP (α,β-meATP) and attenuated by P2X3 receptor antagonists, as well as in P2X3 knockout mice; the P2X3 receptors were localized on suburothelial sensory nerve fibers (Vlaskovska et al., 2001; Carneiro et al., 2014). Prolonged exposure to a desensitizing concentration of α,β-meATP significantly reduced the mechanosensitive activity of the pelvic nerve (Namasivayam et al., 1999). P2X3 knockout mice exhibit reduction of inflammatory pain and marked urinary bladder hyporeflexia with reduced voiding frequency and increased voiding volume, thus suggesting that the P2X3 receptor is involved in mechanosensory transduction underlying both inflammatory pain and physiological voiding reflexes (Cockayne et al., 2000). Subsequently, using P2X2 knockout mice and P2X2/P2X3 double knockout mice, the same group showed that the P2X2 receptor subtype may also be involved in mediating the sensory effect of ATP in the bladder (Cockayne et al., 2005).

The urinary levels of ATP are increased in the patients with neurogenic (Munoz et al., 2012) and non-neurogenic overactive bladders (Silva-Ramos et al.,

2013), bladder outlet obstruction (BOO, Silva-Ramos et al., 2016a), and other lower urinary tract syndromes (Birder et al., 2011). Alterations in urinary ATP are usually accompanied by changes in the density of P2X receptors in the bladder, implying that a concerted action of these changes might have impact in the pathophysiology of lower urinary tract diseases. For instance, patients with BOO exhibit increased urinary ATP (Silva-Ramos et al., 2016a) and overexpress the P2X3 receptor in the bladder urothelium as detected by western blot analysis (Kim et al., 2007). Increased levels of ATP in the bladder are also associated with neurogenic bladder overactivity mediated by activation of P2X3 and P2X2/3 receptors. The same occurred in a rat model of neurogenic detrusor overactivity (Munoz et al., 2012); these authors showed that increased ATP levels strengthened P2X3 and P2X2/3 receptors activation leading to intensification of sensory bladder signals and generation of non-voiding contractions, which might contribute to LUTS.

Disorders of the central nervous system, such as hyperactivity of the spinal cord, which are common in chronic pain states (D'Mello and Dickenson, 2008) may promote a multiple pain mediators release. These mediators (e.g. ATP) can sensitize and affect the expression of P2X3 receptors located in the peripherally and centrally (Paukert et al., 2001; Ramer et al., 2001). In recent years it has been demonstrated that tonic activation of presynaptic P2X3 and P2X2/3 receptors in the spinal cord facilitates the sensory input of the micturition reflex. This has been demonstrated *in vivo* using a novel selective antagonist of these receptors and through activation of the extracellular-signal-regulated kinase (ERK) signaling pathway following a peripheral noxious stimulation (Kaan et al., 2010). These data open avenues for therapeutic approaches to treat various sensory dysfunctions including chronic pain states and potentially, the OAB syndrome, by focusing on targeting the inhibition of sensory purinergic signals (Munoz et al., 2012; Kaan et al., 2010).

4.3.2. P2Y receptors

P2Y receptors are G-protein coupled (metabotropic) receptors, which are categorized into several subfamilies that predominantly couple to (1) G_q ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$) and, therefore, activate phospholipase C (PLC), and (2) G_i ($P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$) that inhibit adenylyl cyclase (AC) (Abbracchio et al., 2006) and regulate voltage-gated ion channels activity (Abbracchio et al., 2006;

Burnstock, 2006). The $P2Y_{11}$ receptor is dually coupled to PLC and AC stimulation (Abbracchio et al., 2006).

Pharmacologically, P2Y receptors can be subdivided into (1) adenine nucleotide-preferring receptors mainly responding to ATP and ADP (human and rodent $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ and human $P2Y_{11}$), (2) uracil nucleotide-preferring receptors (human $P2Y_4$ and $P2Y_6$) responding to either UTP and UDP, respectively, (3) receptors of mixed selectivity (human and rodent $P2Y_2$ and rodent $P2Y_4$), and (4) nucleotide sugar-preferring human $P2Y_{14}$ receptor responding to UDP-glucose and UDP-galactose (Abbracchio et al., 2006; Lazarowski et al., 2003). In summary, the uracil nucleotide, UTP, activates two subtypes of receptors ($P2Y_2$ and $P2Y_4$ receptors), while UDP, previously thought to activate only a single subtype ($P2Y_6$ receptors), is actually known to also activate $P2Y_{14}$ receptors along with the originally designated native agonist UDP-glucose (Carter et al., 2009). Six of the eight known P2Y subtype receptors have been found in the urinary bladder (Table 6).

Table 6. Localization of P2Y receptor subtypes in the urinary bladder.

P2Y RECEPTOR SUBTYPES	LOCALIZATION IN THE BLADDER	SPECIES	REFERENCES
$P2Y_1$	Detrusor; urothelium; suburothelium; epithelial cells	Cat; rat; human	Birder et al., 2003; Carneiro et al., 2014; Säve and Persson, 2010.
$P2Y_2$	Detrusor; epithelial cells	Cat; rat; human	Birder et al., 2003; Chopra et al., 2008; Säve and Persson, 2010.
$P2Y_4$	Urothelium	Cat	Birder et al., 2003.
$P2Y_6$	Detrusor; urothelium	Rat; guinea-pig; mouse	Carneiro et al., 2014; Timóteo et al., 2014; Sin et al., 2006; Yu et al., 2013.
$P2Y_{11}$	Epithelial cells	Human	Säve and Persson, 2010.
$P2Y_{12}$	Detrusor	Mouse	Yu et al., 2013.

Despite the different subtypes of P2Y receptors described, the $P2Y_1$ receptor subtype has been better characterized because it is associated to inhibition of cholinergic neurotransmission in the human bladder (Silva et al., 2011). In addition, cystometry studies demonstrate that activation of the $P2Y_1$ receptor inhibits the tone of the rat detrusor (King et al., 2004; Carneiro et al., 2014).

Interactions between P2Y and P2X receptors have been reported, demonstrating the complexity of the purinergic signaling pathways in the urinary bladder. Yu et al. (2013) showed that activation of the $P2Y_6$ receptor by UDP in detrusor smooth muscle strips from mice modulates P2X1-receptor-mediated contractile responses. These authors hypothesized that synergism between $P2Y_6$ and P2X1 may be relevant under pathological conditions, but this theory was never confirmed or dismissed.

Our group demonstrated that activation of urothelial expressed UDP-sensitive $P2Y_6$ receptors increases the voiding frequency in the anaesthetized rat (Timóteo et al., 2014). This effect is indirectly mediated by ATP released from the urothelium via pannexin-1 hemichannels, which leads to the subsequent activation of P2X3 receptors on sub-urothelial sensory nerve fibers. Interestingly, ATP mediated detrusor contractions, via activation of excitatory P2X1 receptors, may be counteracted by its metabolite, ADP, resulting from its catabolism by ecto-nucleotidases. Formation of ADP in the detrusor layer favors activation of prejunctional inhibitory $P2Y_1$ receptors controlling ACh release from cholinergic nerve endings (Silva et al., 2011).

4.4. ECTO-NTPDASES

As a matter of fact, ATP may have direct effects via activation of P2X purinoceptors, and indirect effects after its enzymatic conversion into ADP and adenosine, which are able to activate P2Y and P1 receptors, respectively. The extracellular hydrolysis of ATP is catalyzed by membrane-bound ecto-nucleotidases, the NTPDases presenting the highest affinity of ATP. These enzymes play very important physiological roles because rapid ATP catabolism results in transient responses of target tissues to ATP and formation of biologically active ATP metabolites (e.g. ADP and adenosine) shifts fast ATP responses to more durable effects operated by metabotropic P2Y and P1 receptors.

Once released into the extracellular space, ATP is hydrolyzed into adenosine diphosphate (ADP) and adenosine monophosphate (AMP) by ecto-nucleoside triphosphate diphosphohydrolases (also known as ecto-nucleotidases, E-NTPDases,

NTPDases), which are then metabolized into adenosine by ecto-5'-nucleotidase (Zimmermann, 2000; Zimmermann et al., 1998).

Four main families of ecto-nucleotidases are characterized: E-NTPDases/Ecto-NTPDases (ectonucleoside triphosphate diphosphohydrolases), nucleotide pyrophosphatase/phosphodiesterases (NPPs), alkaline phosphatases and ecto-5'-nucleotidase (NT5E/ecto-5'-NTPdase). These families differ primarily on their substrate specificities, with E-NTPDases being highly specific for ATP/UTP/ADP/UDP (Robson et al., 2006), while NPPs (Sakagami et al., 2005; Gijsbers et al., 2003) catalyze phosphohydrolysis on a broader range of substrates including lysophospholipids and choline phosphate esters (Robson et al., 2006; Vorhoff et al., 2005; Stefan et al., 2005; Stefan et al., 2006).

The NTPDase family is composed by eight different proteins. Individual NTPDases subtypes differ in functional properties and cellular location. Four of the NTPDases are typically cell surface-located enzymes with an extracellular facing catalytic site (E-NTPDase1, 2, 3, 8). NTPDases 5 and 6 exhibit intracellular localization and undergo secretion after heterologous expression. NTPDases 4 and 7 are entirely intracellularly located, facing the lumen of cytoplasmic organelles (Robson et al., 2006). Plasma membrane bound E-NTPDases can be differentiated according to substrate preference, divalent cation usage and product formation. All E-NTPDases require Ca^{2+} or Mg^{2+} ions in the millimolar range for maximal activity and are inactive in their absence (Zimmermann, 2001; Kukulski et al., 2005). All these enzymes hydrolyse nucleoside triphosphates, including the physiologically active ATP and UTP (Robson et al., 2006). The ATP metabolism and the role of NTPdases are shown in the Figure 4.

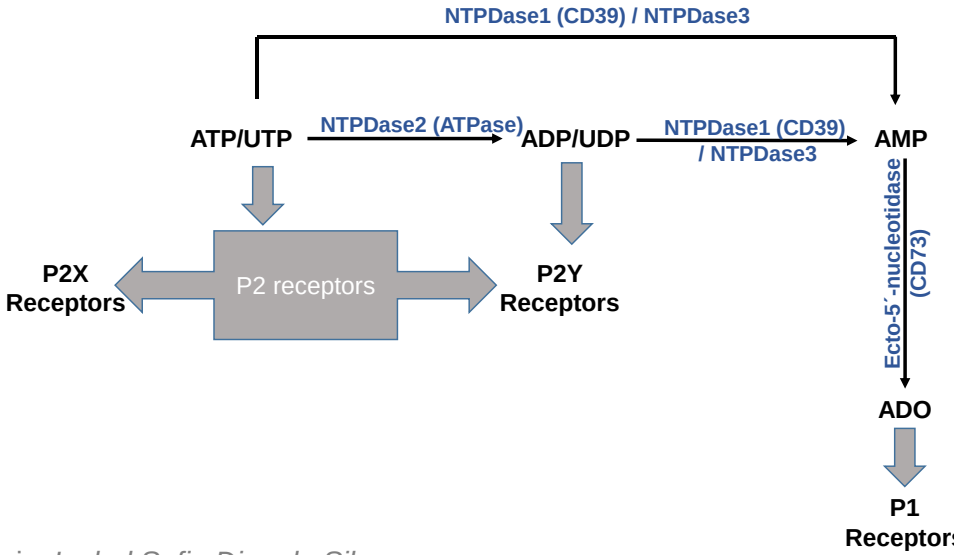


Figure 4. ATP metabolism and role of E-NTPDases. NTPDase1 (CD39 or apyrase) dephosphorylates ATP directly into AMP, with minimal accumulation of ADP. NTPDase2 (ATPase) is a preferential nucleoside triphosphatase hydrolyzing ADP 10 to 15 times less efficiently than ATP, leading to minimal AMP accumulation. NTPDase3 is a functional intermediate between NTPDase1 and NTPDase2. The E-NTPDases cascade can be terminated by ecto-5'-nucleotidase (CD73) with hydrolysis of adenine monophosphate into adenosine**.**

Using molecular biology techniques it was demonstrated that all the eight members of NTPDases, as well as ecto-5'-nucleotidase, are expressed in the mouse bladder. However, of the distribution of NTPDases in the mouse bladder is heterogeneous; E-NTPDase1 is present in the endothelium of blood vessels in the lamina propria and in the detrusor smooth muscle, while E-NTPDase2 is expressed in cells localized to the region of the lamina propria adjacent to the detrusor and surrounding the detrusor muscle bundles (Yu et al., 2011). The same pattern was observed in the rat bladder (Carneiro et al., 2014). E-NTPDase3 is present in the urothelium, occurring on membranes of intermediate and basal epithelial cells, but is absent from the umbrella cells (Yu et al., 2011). The expression and localization of E-NTPDase8 protein in the urothelium was confirmed by immunoblotting and immunofluorescence, respectively. Like the E-NTPDase1 also ecto-5'-nucleotidase was present in the detrusor smooth muscle. Co-localization of these enzymes suggest that ATP-mediated effects may be balanced by fairly rapid adenosine formation, which may fine-tuning control bladder muscle activity during the micturition cycle in the mouse (Yu et al., 2011).

Studies using immunofluorescence confocal microscopy showed that the human urothelium exhibits NTPDase1, NTPDase2 and NTPDase3 in the apical plasma membrane of umbrella cells, whereas the intermediate layer stained positively against E-NTPDase1 and NTPDase2, but no NTPDase3 immunoreactivity was found (Correia-de-Sá, 2010; Silva-Ramos et al., 2015b). The most expressed E-NTPDase2 is the main contributor to the extracellular catabolism of ATP leading to a preferential ADP accumulation with low adenosine formation at the luminal surface of the isolated human urothelium. Despite this, a role for ADP in the urothelium is still missing. Interestingly, tissue elements at or below the basal layer of the urothelium (e.g., blood vessels, myofibroblasts and nerve fibers) and the detrusor smooth muscle layer, were positively stained with NTPDase1, NTPDase2 and ecto-5´-

nucleotidase, which indicates that biosynthesis of adenosine from adenine nucleotides is positioned to favor a more important role of the nucleoside in the suburothelium and the detrusor smooth muscle, namely regulating cholinergic nerve activity (Silva-Ramos et al., 2016b).

Beyond the role of E-NTPDases in the ATP/UTP metabolism and, consequently, the effects through purinergic receptors activation, the function and expression of these enzymes have been implicated in the bladder dysfunctions. In the patients with bladder outlet obstruction (BOO) due to benign prostatic hyperplasia (BPH) it was demonstrated that there is an impairment of E-NTPDase1/CD39 activity in the detrusor smooth muscle leading to the unbalance of extracellular ATP accumulation and endogenous adenosine formation, which might explain the increased neuronal excitation in mucosal-denuded detrusor strips from these patients (Silva-Ramos et al., 2015a). Alterations in the expression of E-NTPDase3 and ecto-5'-nucleotidase/CD73 have also been associated in bladder tumorigenesis in animal models (Rochenbach et al., 2014). The same was observed in human urinary bladder samples from patients with bladder cancer in which an increase of CD73 expression was detected (Wettstein et al., 2015). Ecto-5'-nucleotidase has been proposed as a putative biomarker and/or pharmacological target for bladder cancer (Rochenbach et al., 2014; Wettstein et al., 2015).

4.5. ADENOSINE

As mentioned above, alterations in the activity of E-NTPDases might have implications in endogenous adenosine formation and P1 purinoceptors activation. Extracellular adenosine may originate from the catabolism of released adenine nucleotides (e.g. ATP, cyclic AMP) by E-NTPDases or alkaline phosphatases (Latini and Pedata, 2001; Parkasam et al., 2012). In addition, adenosine may be released as such from cells via equilibrative nucleoside transporters (Hirsh et al., 2007). Inactivation of extracellular adenosine is mainly mediated by cellular uptake and extracellular deamination by adenosine deaminase (ADA). The driving force for adenosine uptake into the cells is its rapid intracellular phosphorylation to AMP by adenosine kinase (AK), which contributes to keep low the cytosolic concentrations of adenosine (Latini and Pedata, 2001).

Distinct mechanisms of adenosine turnover operating at each surface of the uroepithelium have been reported in the urinary bladder; these mechanisms contribute to keep within certain limits the amount of extracellular adenosine (Parkasam et al., 2012). Notwithstanding this, the urothelium can be a site of adenosine biosynthesis and release (Yu et al., 2006). In the rat uroepithelium, adenosine modulates the traffic of vesicles within the cell, which contributes to increase the membrane cell area during bladder filling and to release vesicle-containing signaling mediators. It is highly likely that adenosine might regulate other functions of the bladder mucosa, including ion transport, uroepithelial-afferent nerve signaling, and bladder contraction (Yu et al., 2006). Besides this, adenosine has been shown to negatively modulate stretch-induced ATP release from apical umbrella cells through the activation of A_1 receptor (Dunning-Daves et al., 2013). Taking into consideration that adenosine levels at the luminal site of the urothelium are 10-fold below those found beneath the basal layer, one may hypothesize that adenosine A_1 receptor-mediated facilitation of ATP release plays a minor role in physiological conditions, yet it may contribute to bladder overactivity in disease states once its concentration becomes elevated, like in bladder ischemia-reperfusion and inflammatory cystitis (Silva-Ramos et al., 2016a). Differences in the kinetics of ATP metabolism by ecto-nucleotidases and adenosine biosynthesis between luminal and serosal sides of the urothelium was demonstrated by Silva-Ramos and colleagues, which may explain the dominant role of adenosine in the suburothelium and detrusor smooth muscle layers (Silva-Ramos et al., 2015b). In the urinary bladder, it has also been reported that adenosine partially relaxes pre-contracted urinary bladder detrusor strips exposed to carbachol, ACh, or potassium depolarization in rats, guinea-pigs (Acevedo et al., 1992; Brown et al., 1979; Nicholls et al., 1992; King et al., 1997; Burnstock et al., 1978) and humans (Rubinstein et al., 1998).

4.5.1. P1 receptors

Four types of adenosine receptors have been cloned, A_1, A_{2A}, A_{2B} and A_3; these receptors were initially characterized by their differential coupling to the adenylyl cyclase (AC) / cAMP pathway (Fredholm et al., 2011). All of these receptors are G protein-coupled exhibiting seven transmembrane domains. Adenosine A_1 and A_3 receptors interact with members of the $G_{i/o}$ family and inactive AC to decrease the

production of cAMP, whereas A_{2A} and A_{2B} receptors are coupled to $G_{s/olf}$ and stimulate cAMP production (Klinger et al., 2002; Schulte et al., 2003; Fredholm et al., 2011), resulting in the activation of protein kinase A (PKA) and phosphorylation of the cAMP response element binding protein (CREB) (Fredholm et al., 2011; Cunha, 2001; Paes-de-Carvalho, 2002). In addition, the adenosine A_1 receptor increases phospholipase C (PLC) activity through a pertussis toxin-sensitive G protein. These receptors can couple directly to and inhibit cardiac K^+ channels and types Q, N and P voltage sensitive Ca^{2+} channels (Ali et al., 1991). The A_1 receptor activation can also increase mitogen-activated protein kinase (MAPK) pathway (Shulte and Fredholm, 2000; Dickenson et al., 1998). Activation of the A_{2A} receptor can promote activation of PKC in cAMP-dependent and independent mechanisms (Fredholm et al., 2011; Socodato et al., 2011), contrary to the activation of A_{2B} receptors that can stimulate PKC activity by direct coupling to G_q proteins (Fredholm et al., 2011). The adenosine A_1 and A_{2A} receptors have high affinity for adenosine while the A_{2B} and A_3 receptors show relatively lower affinity for adenosine in rodents (Fredholm et al., 2011).

All these subtypes of adenosine receptors have been shown to exist in the bladder of experimental animals and human beings. RT-PCR analysis showed that that A_{2A} and A_{2B} receptors are more expressed than the A_1 receptor, with the A_3 receptor being the least expressed receptor (Dixon et al., 1996; Owen et al., 2012). Discrepancy between the relative abundance of A_1 and A_2 receptor subtypes in the detrusor (Dixon et al., 1996) may be attributed to their preferential localization on tiny nerve terminals and smooth muscle fibers, respectively (Nicholls et al., 1992; Acevedo et al., 1992).

All four adenosine receptors are expressed in the rat uroepithelium, with the A_1 receptors preferentially located in the apical membrane of the umbrella cells layer, whereas the A_{2A}, A_{2B} and A_3 receptors are localized intracellularly or on the underlying cell layers (Yu et al., 2006). These authors showed that adenosine receptors modulate exocytosis of different molecules in umbrella cells (Yu et al., 2006).

The role of adenosine receptors was also detected in the central control of micturition in rats. Results showed that the inhibitory effect of an A_{2A} receptor antagonist in the spinal cord was enhanced after infusion of acid acetic into the bladder, thus indicating that adenosine exerts an excitatory action via A_{2A} receptor activation in the spinal cord when C-fiber bladder afferents are stimulated by

chemical irritants (Kitta et al., 2014). These findings were corroborated using a rat model of Parkinson's disease showing that the excitatory drive mediated by adenosine A_{2A} receptors on bladder overactivity was operated at a supraspinal site (Kitta et al., 2014); bladder overactivity in these animals were also suppressed by A_{2A} receptors blockage. The involvement of A_1 receptors in the central control of micturition has also been observed. Data showed that activation of inhibitory adenosine A_1 receptors in the brain may counteract nociceptive inputs from the bladder. Moreover, activation of the A_1 receptor also exerts a peripheral inhibitory effect on micturition (Kitta et al., 2014). These findings suggest that adenosine A_1 receptor agonists and A_{2A} receptor antagonists might be effective for the treatment of overactive bladder (Kitta et al., 2014; Kitta et al., 2012).

Previous studies showed that A_1 and A_{2A} receptors are present in the human detrusor, while immunoreactivity against A_{2B} and A_3 receptors was vestigial. Our group demonstrated that the adenosine A_1 receptor is predominantly localized in cholinergic nerve terminals and that this receptor subtype is overexpressed in bladder strips from patients with BOO due to benign prostatic hyperplasia (BPH) (Silva-Ramos et al., 2015a). Functional data show that adenosine significantly decreases [^{3}H]ACh release from bladder strips of BOO patients and this effect depends on A_1 receptors activation (Silva-Ramos et al., 2015a). Interestingly, the adenosine inhibitory effect on [^{3}H]ACh release was significantly higher in BPH patients than in control individuals (Silva-Ramos et al., 2015a). The immunolocalization studies showed that A_1 and A_{2A} receptors are the most expressed receptors in the human detrusor, whereas immunoreactivity of A_{2B} and A_3 receptors is less evident. Co-localization studies showed a differential distribution of the A_1 receptor, which is localized preferentially on VAChT positive cholinergic nerves, whereas the A_{2A} receptor is diffusely expressed on smooth muscle fibers of the human detrusor (Silva-Ramos et al., 2015a). On the other hand, the activation of A_{2A} receptors with a selective agonist, CGS21680C, didn´t have a significantly effect in the evoked-[^{3}H]ACh release. Thus suggesting that adenosine via A_1 receptors activation might have a dominant effect in to reducing detrusor hyperactivity in the human bladder (Silva-Ramos et al., 2015a). Data obtained in the human bladder agree with the hypothesis raised in rodents (e.g. bladder overactivity due to acetic acid irritation) concerning the therapeutic potential of adenosine A_1 receptor activation to reduce bladder overactivity (Kitta et al., 2014). In patients with BOO due

BPH, was observed an impairment of ecto-NTPDase1 activity that unbalances extracellular ATP accumulation and endogenous adenosine formation leading to increased neuronal excitation in detrusor from these patients (Silva-Ramos et al., 2015a). While extracellular ATP accumulation may contribute to hyperexcitation of suburothelial nerve afferents via P2X3 receptors and to detrusor reactivity via P2X1 subunit-containing receptors. Deficits in adenosine formation may also play a role in generating symptoms of bladder dysfunction in BPH patients (Silva-Ramos et al., 2015a). Thus, the loss of the inhibitory tone exerted by prejunctional A_1 receptors on ACh release from stimulated cholinergic nerves in the detrusor may be a target for therapeutic intervention of bladder dysfunctions associated with outflow obstruction (Silva-Ramos et al., 2015a).

Recent evidences based on electrophysiologic and myographic recordings corroborate the theory that activation of prejunctional A_1 adenosine receptors may provide a mean to dampen aberrant neurotransmission to the detrusor in human and murine bladders (Searl et al., 2016), supporting a putative role for A_1 receptor agonists in the treatment of LUTS.

5. Adrenergic system

The bladder filling is primarily controlled by sympathetic inputs, whereas voiding is under parasympathetic influence. During the filling, distension of the detrusor muscle in the body of the bladder is achieved by suppression of the excitatory efferent parasympathetic influence through neural reflex and the concomitant closure of the bladder outlet through sympathetic-mediated tonic contraction of the base of the bladder and the urethra (Applebaum et al., 1980; Maggi et al., 1987, 1988; Elbadawi, 1988). Postganglionic sympathetic neurons innervating the bladder release norepinephrine (also denominated as noradrenaline, NA) which binds to both α- and β-adrenoceptors (de Groat and Booth, 1980; Maggi and Meli, 1982). Sympathetic stimulation of the LUT can lead to both excitatory and inhibitory effects. The highest density of adrenergic nerves is present in the urethra, the bladder neck and the bladder body (de Groat and Yoshimura, 2001). In the urethra and the bladder neck, the smooth muscle cells express predominantly α_1-receptors which are responsible for generating muscle tone and occluding the urethral orifice

during the storage phase of the micturition cycle (de Groat and Yoshimura, 2001). In the bladder body, adrenergic fibers are found manly in the smooth muscle bundles. Relaxation of the smooth muscle is mediated by β-adrenergic receptors (Andersson, 1993). NA has revealed to be an effective inhibitor of bladder muscle strip contractions (Âmark et al., 1986).

The adrenergic receptors were differentiated pharmacologically into α- and β-adrenergic receptors for the first time in 1948 by Ahlquist (Ahlquist, 1948). Several years later the adrenoceptors were distinguished into four subtypes using adrenergic agonists and antagonists. These types are: α_1, α_2, β_1 and β_2 adrenergic receptors (Lands et al., 1967; Ablad et al., 1974). Later on, a new subtype of β-adrenergic receptor was characterized, the β_3-adrenoceptor.

Actually three different β-adrenoceptors subtypes have been cloned and characterized using pharmacological methods (Bylund et al., 1994). The β_3-adrenoceptor was cloned in 1989 and has been intensively studied because of their involvement in glucidic and lipidic metabolism. β-adrenoceptors are G protein-coupled receptors (Strosberg, 1993) exhibiting seven transmembrane domains (Coman et al., 2009) spanning the lipid bilayer.

The mRNA of all three β-adrenoceptor isoforms (β_1, β_2, β_3) has been identified in the detrusor (Fujimura et al., 1999; Seguchi et al., 1998); 97% of the β-adrenoceptor mRNA found in the detrusor is for the β_3-adrenergic receptor subtype, while only 1.5% and 1.4% refers to β_1- and β_2-adrenoceptors mRNAs, respectively (Nomiya and Yamaguchi, 2003; Michel and Vrydag, 2006). Immunolocalization studies demonstrate that β-adrenoceptors are present throughout the bladder. The β_1-adrenoceptor is present in the urothelium and though in lesser amounts in ICCs and detrusor smooth muscle fibers. The β_2-adrenoceptor is located in the urothelium, ICCs and detrusor muscle fibers, but its distribution is heterogeneous being the urothelium the most enriched region and the detrusor muscle the less labeled region. The β_3-adrenoceptor is expressed in higher amounts in interstitial cells, whereas lesser amounts are found in the urothelium (Limberg et al., 2010; Otsuka et al., 2013), detrusor smooth muscle fibers (Otsuka et al., 2013), and cholinergic nerve terminals innervating the detrusor (Coelho et al., 2017). Receptor expression studies do not perfectly match functional data using selective β-adrenoceptors agonists; the β_3-adrenoceptor seems to be the most relevant receptor subtype causing human detrusor relaxation (Igawa et al., 1998; Svalo et al., 2013). The noradrenaline effect

observed in the bladder body is mainly attributed to the β_3-adrenoceptor, while the α_1-adrenoceptor seems to be less important (Chou et al., 2003). Unlike other receptor subtypes, like purinergic receptors, β_3-adrenoceptors expression is not affected by age of the individuals (Limberg et al., 2010).

Regarding ICCs present in the bladder wall, it was demonstrated that a population of these cells have the machinery to store, release and respond to catecholamines. Thus, it is highly possible that these cells are part of a β-adrenergic system operating in the bladder wall beyond noradrenergic nerves and the receptors present in the detrusor smooth muscle. The ICC-related β-adrenergic system is possibly linked to the generation of non-voiding spontaneous microcontractions and may be involved in mechanisms underpinning bladder sensation (Persyn et al., 2016). β_3-adrenergic receptor agonists may increase bladder capacity and inhibit the bladder instability associated with hyperreflexia and detrusor hypertrophy (Woods et al., 2001). These receptors are coupled to the stimulatory G protein (G_s) and its agonists have the ability to activate AC leading to increases in intracellular cAMP and, thereby, protein kinase A activation causing relaxation of the bladder smooth muscle (Tyagi et al., 2009; Wallukat, 2002; Jóźwiak-Bębenista et al., 2016). On the other hand, is questioned the role of cAMP in β-adrenoceptors mediates relaxation because inhibitors of AC or PKA had limited effect on rat detrusor relaxation, suggesting that other unknown pathways are involved (Frazier et al., 2005).

Interestingly, the AC/cAMP intracellular pathway is affected by hypoxic conditions (Jóźwiak-Bębenista et al., 2016), which have been implicated in LUTS, thus affecting the therapeutic success of β_3-adrenergic receptor agonists.

6. Lower urinary tract symptoms

The term “lower urinary tract symptoms (LUTS)” was introduced 13 years ago by Abrams (Abrams, 1994). LUTS designate a number of symptoms regarding storage (irritative), voiding (obstructive), and postmicturition symptoms (Abrams et al., 2002). According with the International Continence Society (ICS), voiding symptoms include slow stream, splitting / spraying, hesitancy, intermittency, straining and terminal dribble (Abbrams et al., 2002), while storage symptoms are primarily

related to overactive bladder (OAB), like urgency with or without urgency incontinence, usually with frequency and nocturia (Abrams et al., 2002).

In a large population-based survey known as the EPIC study, the prevalence of any voiding symptom was 26% among men and 20% among women. However, this percentage was higher when looking for the prevalence of any storage symptoms, 51% and 60% among men and women, respectively (Irwin et al., 2006). The prevalence of LUTS suggestive of OAB was 10.8% in men and 12.8% in women (Irwin et al., 2006). In Portugal, the prevalence of OAB in people with 40 years of age or older was reported to be of 29.4% in women and of 35.1% in men (Correia et al., 2009).

It is clearly recognized that OAB symptoms have a negative impact in the quality of life of both men and women, reducing emotional wellbeing and productivity (Currie et al., 2006; Irwin et al., 2006a; Stewart et al., 2003). OAB without urge incontinence is more common in men, whereas association with incontinence has a female predominance. The frequency of associated female incontinence increases with age but it significantly increases after approximately 45 years of age. In contrast, male-associated incontinence increases with age but rises sharply after approximately 65 years of age (Helfand et al., 2009).

Men with 50 years of age or older who complaint predominantly of voiding symptoms are typically considered to have LUTS, which is suggestive of bladder outlet obstruction (BOO). Benign prostatic hyperplasia (BPH) is a strictly histological diagnosis and may result in BOO with postmicturition symptoms that include the feeling of incomplete emptying and post-micturition dribble (Abbrams et al., 2002). Histological BPH was never observed in men under the age of 30 years. Approximately half of the men in their sixth decade of life exhibit histological evidence of BPH. Almost 90% of men develop histologic BPH by the ninth decade of life (Lepor, 2004).

OAB and BPH, contribute substantially to societal burden. A health economic model estimated that 20 million people aged older than 40 years of age experienced symptoms of OAB in 2000 (Irwin et al., 2006); at the moment, it is estimated that an excess of 100 million men and women are afflicted with bothersome LUTS due to BPH and OAB, respectively. The prevision for the year 2020 is that more than 25% of the population worldwide will be affected with by LUTS (Irwin et al., 2006).

7. Current treatments of LUTS

The difficulty in treating LUTS begins in the suboptimal knowledge about the pathophysiology of these conditions. Interestingly, all these disease conditions have a common feature as they are associated with increased release of ATP from bladder urothelial cells above the levels released normally in response to bladder distension during urine storage (Fergunson et al., 1997; Vlaskovska et al., 2001). Augmentation of urinary ATP levels was verified in patients with interstitial cystitis / bladder pain syndrome (IC/BPS) (Sun et al., 2001; Fry et al., 2014), but also in women with idiopathic OAB (Silva-Ramos et al., 2013) and men with LUTS due to BPH (Silva-Ramos et al., 2016a). This led to the conclusion that urinary ATP measurements may function as a highly-sensitive biomarker of bladder overactivity (Silva-Ramos et al., 2013; 2016a). On the other hand, the increase in the expression of P2X3/P2X2/3 receptors in the detrusor smooth muscle, urothelium and afferent terminals from animal models and patients with LUTS, prompted for studies to investigate these receptors as putative therapeutic targets (Burnstock, 2017). AF-219, a P2X3/P2X2/3 antagonist that is metabolically stable and orally bioavailable, is being investigated as a treatment for urinary tract dysfunctions (Ford and Cockayne, 2011). Manipulation of other purinergic receptors, such as P2X1 and P2X7, that are impaired in LUTS has also been investigated as putative new drugs targets to improvement the LUTS (Burnstock et al., 2017).

Anticholinergic therapy is more common and anti-muscarinic drugs are first line treatment of LUTS, predominantly of the OAB type. More recently, it has been approved the clinical use of β_3-adrenoceptors agonists (mirabegron) to treat OAB syndromes (Andersson, 2013; Andersson et al., 2013). Other drugs, such as the phosphodiesterase 5 inhibitor (tadalafil) (Lythgoe et al., 2013), and the blocking of afferent and efferent nerves with botulinum toxin (Magera et al., 2014; Andersson, 2013; Laerence et al., 2010; Smith et al., 2005; Chancellor et al., 2008), were also introduced. There are LUTS treatment options not involving drugs; these are intermittent catheterization, sacral neuromodulation and posterior tibial nerve stimulation (Chua et al., 2015).

7.1. ANTICHOLINERGIC DRUGS

The mainstay of treatment of OAB is the use of anticholinergics, which exert their action by blocking the muscarinic receptors located in the bladder smooth muscle (Abrams et al., 2006b). Although muscarinic M_2 receptors dominate M_3 receptors in the bladder in a 3:1 ratio, the muscarinic M_3 receptors seem to be the most important muscarinic receptor for the detrusor contraction (Abrams et al., 2006b; Chess-Williams et al., 2001). The muscarinic M_2 receptors located in the bladder may play a role in the detrusor contraction by reversing β-adrenoceptor-mediated smooth muscle relaxation, indirectly enhancing M_3 receptor mediated contractions, or increasing urgency sensations (Skaskin et al., 2011; Hegde et al., 1997; Yamanishi et al., 2000; Daly et al., 2010). Muscarinic M_2 receptors are also located in the heart and modulate the heart pacemaker activity and atrioventricular conduction. Blockage of the M_2 receptors, in addition to the inhibitory effect on the detrusor contractions through M_3 pathways, also results in an increase in heart rate (Harvey and Belevych, 2003). Antimuscarinic drugs differ in their pharmacological profile at the five distinct human muscarinic receptors (Andersson et al., 2011). Essentially, tolterodine, fesoterodine, propiverine and trospium, do not discriminate among the five muscarinic receptor subtypes. Oxybutynin and solifenacin possess a marginal selectivity for M_3 receptors over the muscarinic M_2/M_3 subtypes receptors, whereas darifenacin has a high degree of selectivity for muscarinic M_3 receptors over the muscarinic M_2/M_4 receptors subtypes. Theoretically, in terms of the increase in heart rate, OAB antimuscarinics having selectivity for muscarinic M_3 over M_2 receptors must be advantageous compared to non-selectivity drugs (Andersson et al., 2011). The M_3 receptor subtype mediating bladder contractions is also located in the salivary glands, gastrointestinal smooth muscle, and ciliary and iris sphincter muscles. Therefore, blockade of this receptor results in side effects common to most anti-muscarinic drugs, such as dry mouth, constipation, and blurred vision (Chapple et al., 2008; Novara et al., 2008; Kessler et al., 2011; Meek et al., 2011).

Despite the high intake of medications with anticholinergic properties by the community-dwelling elderly population, the effects on cognitive decline and dementia have rarely been evaluated. The risk of incident dementia over a 4-years follow-up period was also increased in continuous users (hazard ratio (HR), 1.65; 95% CI, 1.00-2.73) but not in those who discontinued the use of anticholinergic drugs (HR,

1.28; 95% CI, 0.59-2.76). Elderly people taking anticholinergic drugs were at an increased risk of cognitive decline and dementia. Discontinuing anticholinergic treatment was associated with a decreased risk (Carrière et al., 2009). Adherence to anticholinergic therapy in OAB has been shown to be as low as 20% after 6 months of follow-up in real-life practice (Kelleher et al., 1997).

7.2. β_3-ADRENOCEPTOR AGONISTS

Whereas anti-muscarinics bind to muscarinic receptors of the urinary bladder and inhibit involuntary bladder contractions, stimulation of β_3-adrenoceptors in the detrusor muscle involves bladder relaxation during the filling phase, thus improving the bladder storage capacity (Igawa et al., 1999; Fujimura et al., 1999). Herein lays the potential of the drugs that act as β_3-adrenoceptor agonists to treat the OAB symptoms (Kumar et al., 2003).

In June of 2012, mirabegron, a selective β_3-adrenergic receptor agonist was approved for the treatment of OAB with symptoms of urge urinary incontinence in the United States (US) under the branded name *Myrbetriq*® (Astellas Pharma US Inc.). In July 2011, the same drug received approval for its use in Japan (under the name of *Betanis*®) and by 2013 the European Commission approved its use in Europe (under the name of *Betmiga*®).

Mirabegron is a first-in-class therapeutic agent with a novel mechanism of action for the treatment of OAB. Mirabegron improves the storage capacity of the bladder, extending the duration of the storage phase, with potentially greater tolerability compared to the anti-muscarinic agents (Package, 2012). In clinical studies, the most frequently reported adverse effects occurring in patients receiving treatment with mirabegron include hypertension, nasopharyngitis, urinary tract infection, headache, constipation, upper respiratory tract infection, arthralgia, and tachycardia (Package, 2012). The potential for mirabegron to non-selectively stimulate β_3-receptors in other types of smooth muscle may lead to both increased blood pressure and heart rate. As a result, mirabegron may not be suitable for patients with severe uncontrolled hypertension or tachycardia prior to use, orthostatic hypotension, or any other significant cardiac condition that may predispose to arrhythmias (Bridgeman et al., 2013). However, more than 50% of the patients had

previously discontinued anti-cholinergics medication for OAB, thus allowing us to obtain data on the effectiveness of mirabegron in patients already treated with anticholinergics (Angulo et al., 2013), once time, that the action mechanism of mirabegron is unknown.

7.3. BOTULINUM TOXIN

In addition to the therapies mentioned above, botulinum toxin (BoNT) is also being used for the treatment of OAB. This therapeutic alternative is used for patients whose symptoms are refractory to conventional therapy (e.g. anti-cholinergic drugs) (Harris and Rissolo, 2016; Jhang and Kuo, 2016). The BoNT has two different sites of action: it reduces the release of both ACh and ATP from parasympathetic nerves (Lawrence et al., 2010), and, on the other hand, it reduces the release of ACh and ATP from urothelial cells, reducing, consequently, the stimulatory drive of the voiding reflex (Smith et al., 2005; Chancellor et al., 2008). The BoNT treatment also alter the expression of P2 and other receptors. Four weeks after BoNT injections was demonstrated the decreased expression of P2X3 and TRPV1 receptors in the bladder mucosa and suburothelial nerves fibers, respectively from patients with overactivity detrusor (Apostolidis et al., 2005). Additionally, the decline in P2X3 receptors was significantly correlated with the reduction of urgency episodes four and sixteen weeks after botulin toxin injection (Apostolidis et al., 2005). Therefore, it appears that the clinical efficacy of BoNT relies not only on the blockade of transmitter release from efferent nerves, but also on its action on urothelial sensory mechanisms. The BoNT, in particulary the onabotulinumtoxin-A type, provided greater relief of OAB symptoms compared with most other licensed doses of anticholinergics and mirabegron in the network (Drake et al., 2017).

V. AIMS

The main objective of this thesis was to investigate the role of the purinome (purines releasing sites, enzymatic breakdown and purinoceptors activation) to control hyperactivity of the human urinary bladder prompting for new pharmacological targets to treat overactive bladder (OAB) syndromes.

Our specific objectives were:

(1) To evaluate in parallel changes in the release of ATP and ACh from the urothelium of patients with bladder outlet obstruction (BOO) compared to control individuals;

(2) To study the role of UDP-sensitive $P2Y_6$ receptors in the human urothelium from patients with BOO due benign prostatic hyperplasia (BPH) compared to control men;

(3) To investigate the role of ADP-sensitive receptors in the control of excitability produced by P2X2/3 receptors activation on evoked [^{3}H]ACh release from cholinergic nerves in the bladder of control individuals and obstructed patients due to BPH.

(4) To explore the participation of purines in the therapeutic effect of β_3-adrenoceptor agonists in human and rat urinary bladders;

(5) To investigate the intracellular signaling pathways linking β_3-adrenoceptors activation and adenosine overflow from human and rat detrusor smooth muscle fibers.

VI. ORIGINAL RESEARCH ARTICLES

The results presented in this thesis were published / submitted for publication as original research full papers, as follows:

2015 - "**Activation of $P2Y_6$ receptors facilitates nonneuronal adenosine triphosphate and acetylcholine release from urothelium with the lamina propria of men with bladder outlet obstruction**"

Silva I, Ferreirinha F, Magalhães-Cardoso MT, Silva-Ramos M, Correia-de-Sá P. *Journal of Urology*, 194: 1146-1154. doi: 10.1016/j.juro.2015.05.080.

- "**Research Highlight – BPH – $P2Y_6$ blockade might help control bladder storage symptoms**" by Rebecca Kelsey
  Nature Reviews Urology, 12 (7) -358, doi: 10.1038/nrurol.2015.

2017 - **"Inhibition of cholinergic neurotransmission by β_3-adrenoceptors depends on adenosine release and A_1 receptors activation in human and rat urinary bladders"**

Silva I, Costa AF, Moreira S, Ferreirinha F, Magalhães-Cardoso MT, Calejo I, Silva-Ramos M, Correia-de-Sá P.

American Journal of Physiology – Renal Physiology, 313(2): F388-F403, doi: 10.1152/ajprenal.00392.2016.

- "**Editorial Focus – Do β_3-adrenoceptor agonists cause urinary bladder smooth muscle relaxation by inhibiting acetylcholine release? Focus on "Inhibition of cholinergic neurotransmission by β_3-adrenoceptors depends on adenosine release and A_1 receptors activation in human and rat urinary bladder**" by Katerina Okeke, Stavros Gravas, Martin Michel.
  American Journal of Physiology – Renal Physiology, 313: F859-F861, doi: 10.1152/ajprenal.00215.2017.
- "**Words of Widsom – Re: Inhibition of Cholinergic Neurotransmission by β_3-adrenoceptors depends on adenosine release and A_1 receptors activation in human and rat urinary bladder**" by Karl-Erik Andersson.
  European Urology, 72 (4): 650-655, doi: 10.1016/j.eururo.2017.05.046.

2018 - "Balance between excitatory P2X2/3 and inhibitory $P2Y_{12}$ receptors in men with prostatic bladder obstruction to control cholinergic nerve activity"
Silva I, Ferreirinha F, Magalhães-Cardoso MT, Silva-Ramos M, Pelletirer J, Sévigny J, Correia-de-Sá P.
Manuscript in preparation

2018 - "Cyclic AMP activation of the exchange protein EPAC mediates β_3-adrenoceptor inhibition of cholinergic neurotransmission in human and rat urinary bladders"
Silva I, Magalhães-Cardoso MT, Ferreirinha F, Moreira S, Costa AF, Silva-Ramos M, Correia-de-Sá P.
Manuscript in preparation

Data from the following research papers co-authored by the proponent were also used and/or discussed in this thesis:

2013 – "Urinary ATP may be a dynamic biomarker of detrusor overactivity in women with overactive bladder syndrome"
Silva-Ramos M*, **Silva I***, Oliveira O, Ferreira S, Reis MJ, Oliveira JC, Correia-de-Sá P.
Plos One, 8 (5): e64696, doi: 10.1371/journal.pone.0064696.

2014 – "Activation of $P2Y_6$ receptors increases the voiding frequency in anaesthetized rats by releasing ATP from the bladder urothelium"
Carneiro I*, Timóteo MA*, **Silva I**, Vieira C, Baldaia C, Ferreirinha F, Silva-Ramos M, Correia-de-Sá P.
British Journal of Pharmacol, 171, 3404-3419, doi: 10.1111/bph.12711.

"ATP release via pannexin-1 hemichannels mediates bladder overactivity triggered by urothelial $P2Y_6$ receptors"
Timóteo MA, Carneiro I, **Silva I**, Noronha-Matos JB, Ferreirinha F, Silva-Ramos M, Correia-de-Sá P.
Biochemistry Pharmacology, 87(2): 371-9, doi: 10.1016/j.bcp.2013.11.007.

2015 – "Impairment of ATP hydrolysis decreases adenosine A_1 receptor tonus favoring cholinergic nerve hyperactivity in the obstructed human urinary bladder"

Silva-Ramos M*, **Silva I***, Faria M, Magalhães-Cardoso MT, Correia J, Ferreirinha F, Correia-de-Sá P.

Purinergic Signaling, 11, 595-606, doi: 10.1007/s11302-015-9478-z.

2016 – "Increased urinary adenosine triphosphate in patients with bladder outlet obstruction due to benign prostate hyperplasia"

Silva-Ramos M, **Silva I**, Oliveira JC, Correia-de-Sá P.

Prostate, 76 (15): 1353-63, doi: 10.1002/pros.23207.

BOOK CHAPTERS

2015 - **"Urinary Bladder Disorders: Is Adenosine Friend or Foe?"**

M. Silva-Ramos*, **I. Silva***, M. Faria, M.T. Magalhães-Cardoso and P. Correia-de-Sá.

In *Adenosine Receptors: Pharmacology, Functions and Therapeutic Aspects*. Ed. K. Warrick, Nova Science Publishers, Inc (USA). Chapter 4, pp. 115-142. ISBN: 978-1-63463-471-7.

2016 – "Overactive bladder (OAB): Is there a place for drugs targeting the purinergic cascade?"

M. Silva-Ramos, **I. Silva**, F. Ferreirinha, M. Faria and P. Correia-de-Sá.

In *Overactive Bladder (OAB): Prevalence, Risk Factors and Management*. Ed. Elvira Larson, Nova Science Publishers, Inc (USA). Chapter 6, pp. 127-156. ISBN: 978-1-63485-033-9.

* Authors contributing equally to the work.

PAPER 1

Journal of Urology, 2015, 194: 1146-1154.
DOI: 10.1016/j.juro.2015.05.080.

Activation of $P2Y_6$ Receptors Facilitates Nonneuronal Adenosine Triphosphate and Acetylcholine Release from Urothelium with the Lamina Propria of Men with Bladder Outlet Obstruction

Isabel Silva[1,2], Fátima Ferreirinha[1,2], Maria Teresa Magalhães-Cardoso[1,2], Miguel Silva-Ramos[1,2,3] and Paulo Correia-de-Sá[1,2]*

[1]Laboratório de Farmacologia e Neurobiologia, [2]Center for Drug Discovery and Innovative Medicines, Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto and [3]Serviço de Urologia, Centro Hospitalar do Porto, Porto, Portugal

* Corresponding author: farmacol@icbas.up.pt

Contribution of authors: PCS supervised the project, designed the experiments, and analyzed data. IS, and PCS wrote the paper. IS and MTMC performed and analyzed data from enzymatic kinetic experiments by HPLC. IS performed and analyzed data from acetylcholine release experiments. IS and FF performed immunofluorescence staining and confocal microscopy observations. MSR recruited the patients and collected the bladder samples. IS, FF, MTMC, MSR and PCS interpreted data, discussed the clinical implications, and commented on the manuscript at all stages.

ABSTRACT

Purpose: Deregulation of purinergic bladder signaling may contribute to persistent detrusor overactivity in patients with bladder outlet obstruction. Activation of uridine diphosphate sensitive $P2Y_6$ receptors increases voiding frequency in rats indirectly by releasing adenosine triphosphate from the urothelium. To our knowledge this mechanism has never been tested in the human bladder.

Materials and Methods: We examined the role of the uridine diphosphate sensitive $P2Y_6$ receptor on tetrodotoxin insensitive nonneuronal adenosine triphosphate and [^{3}H]acetylcholine release from the human urothelium with the lamina propria of control organ donors and patients with benign prostatic hyperplasia.

Results: The adenosine triphosphate-to-[^{3}H]acetylcholine ratio was fivefold higher in mucosal urothelium/lamina propria strips from benign prostatic hyperplasia patients than control men. The selective $P2Y_6$ receptor agonist PSB0474 (100 nM) augmented by a similar amount adenosine triphosphate and [^{3}H]acetylcholine release from mucosal urothelium/lamina propria strips from both groups of

individuals. The facilitatory effect of PSB0474 was prevented by MRS2578 (50 nM) and by carbenoxolone (10 μM), which block $P2Y_6$ receptor and pannexin-1 hemichannels, respectively. Blockade of P2X3 (and/or P2X2/3) receptors with A317491 (100 nM) also attenuated release facilitation by PSB0474 in control men but not in patients with benign prostatic hyperplasia. Immunolocalization studies showed that $P2Y_6$, P2X2 and P2X3 receptors were present in choline acetyltransferase positive urothelial cells. In contrast to $P2Y_6$ staining, choline acetyltransferase, P2X2 and P2X3 immunoreactivity decreased in the urothelium of benign prostatic hyperplasia patients.

Conclusions: Activation of $P2Y_6$ receptor amplifies mucosal adenosine triphosphate release underlying bladder overactivity in patients with benign prostatic hyperplasia. Therefore, we propose selective $P2Y_6$ receptor blockade as a novel therapeutic strategy to control persistent storage symptoms in obstructed patients.

Key Words: urinary bladder neck obstruction, urothelium, acetylcholine, adenosine triphosphate, purinoceptor $P2Y_6$.

INTRODUCTION

Changes in the bladder sensory system during urine storage may contribute to detrusor overactivity in patients with BOO. The urothelium has a key sensory role during bladder filling by releasing chemical mediators, including ACh and ATP, from nonneuronal stores (Yoshida et al., 2010). Targeting sensory nerve afferents by signaling molecules released from the urothelium may be exaggerated under pathological conditions, leading to increased bladder sensation and detrusor overactivity. Thus, we hypothesized that the urothelium may be a key factor in the mechanism through which BOO associated with BPH causes lower urinary tract symptoms (Mirone et al., 2017). Long-term changes in nonneuronal ACh and ATP release from the urothelium may explain why many patients undergoing obstruction relief procedures such as prostatectomy still report persistent storage symptoms.

While neuronal release of ACh acting mainly on detrusor smooth muscle muscarinic M_3 receptors promotes bladder emptying (Chess-Williams et al., 2001), nonneuronal ACh synthesis and release from the urothelium may occur during the

storage phase (Winder et al., 2014). Exposure of urothelium, afferent nerves and detrusor smooth muscle simultaneously to ACh and ATP may change under pathological conditions (Ochodnicky et al., 2013). Therefore, the precise mechanisms and sites of action of muscarinic receptor antagonists used to treat overactive bladder symptoms remain elusive (Sellers and Chess-Williams, 2012). Moreover, an increase in atropine resistant purinergic tone through the abnormal production, release and metabolism of ATP or altered expression of various P2 purinoceptors is a common feature of many urological diseases (Ruggieri, 2006; Sjöogren et al., 1982; Palea et al., 1993; Yoshida et al., 2004). Mounting evidence suggests that ATP is released predominantly from the bladder urothelium in response to mechanical (distension) and chemical (capsaicin, ACh and, bradykinin) stimulation (Khera et al., 2004; Wang et al., 2005). Urothelial ATP release is augmented in patients with increased bladder sensation and voiding frequency (Sun and Chai, 2006; Silva-Ramos et al., 2013; Sun et al., 2002).

In contrast to the compelling evidence of the extracellular signaling role of ATP, the hypothesis that uracil nucleotides also have autocrine/paracrine actions has only recently gained experimental support. Our group reported that activation of the UDP sensitive $P2Y_6$ receptor increased voiding frequency in anesthetized rats indirectly by releasing ATP from the urothelium via pannexin-1 hemichannels (Timóteo et al., 2014) and subsequent activation of P2X3 receptors on suburothelial nerve afferents (Carneiro et al., 2014). In the bladder ionotropic P2X3 receptors can function as homomultimeric P2X3 and heteromultimeric P2X2/3 channels (Cockayne et al., 2005). Involvement of pannexin-1 hemichannels provides a direct pathway for mechanically induced ATP release and for ATP induced ATP release through interaction with P2 purinoceptors (Negoro et al., 2014). The precise transport mechanisms involved in ATP release are still under active debate. There is evidence for exocytotic vesicular release of ATP from urothelial cells while nonvesicular ATP release may also be mediated by the activation of stretch, voltage and/or ligand gated ion channels and receptors, mitochondrial porins and ATP binding cassette transporters (Winder et al., 2014). We examined the role of $P2Y_6$ receptor on nonneuronal ATP and [^{3}H]ACh release from mucosal U/LP strips from control organ donors and patients with BPH using highly selective agonists and antagonists to understand the storage symptoms remaining in patients after obstruction relief procedures. Given that morphological and functional abnormalities in the epithelium,

smooth muscle fibers, extracellular matrix and neuronal network are common in the bladder of patients with BPH (Mirone et al., 2007), we also evaluated changes in the presence of ChAT (the enzyme responsible for ACh synthesis) and the P2 receptor subtypes metabotropic $P2Y_6$, and ionotropic P2X2 and P2X3 in the human bladder mucosa by confocal microscopy.

MATERIAL AND METHODS

Human Bladder Samples

Samples were collected from the anterior wall of the bladder dome of 50 consecutive patients with a mean ± SEM age of 70 ± 2 years with BPH who underwent transvesical prostatectomy and 26 male organ donors with a mean age 55 ± 3 years at the time of organ harvest for transplantation. Samples were immediately placed at 4ºC to 6ºC in mannitol transplantation solution at 400 mOsm/kg (M-400) not supplemented with ATP or adenosine (4.190 gm 100 ml^{-1} mannitol, 0.205 gm 100 ml^{-1} KH_2PO_4, 0.970 gm 100 ml^{-1} $K_2HPO_4.3H_2O$, 0.112 gm 100 ml^{-1} KCl and 0.084 gm 100 ml^{-1} $NaHCO_3$, 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 Centro Hospitalar do Porto and Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto. All patients with BPH provided signed informed consent to use the biological material. The investigation conformed to the principles outlined in the Declaration of Helsinki.

ATP and [^{3}H]ACh Release Quantification

Human mucosal U/LP strips can be easily separated from the underlying smooth muscle layer by blunt dissection (Kumar et al., 2004). Absence of the detrusor smooth muscle layer was confirmed by confocal microscopy (Figure 5).

Mucosal strips were mounted in 3 ml perfusion chambers heated to 37ºC and superfused with gassed (95% O_2 and 5% CO_2) Tyrode's solution (pH 7.4) containing (mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1, NaH_2PO_4 0.4, $NaHCO_3$ 11.9, glucose 11.2 and choline 0.001.

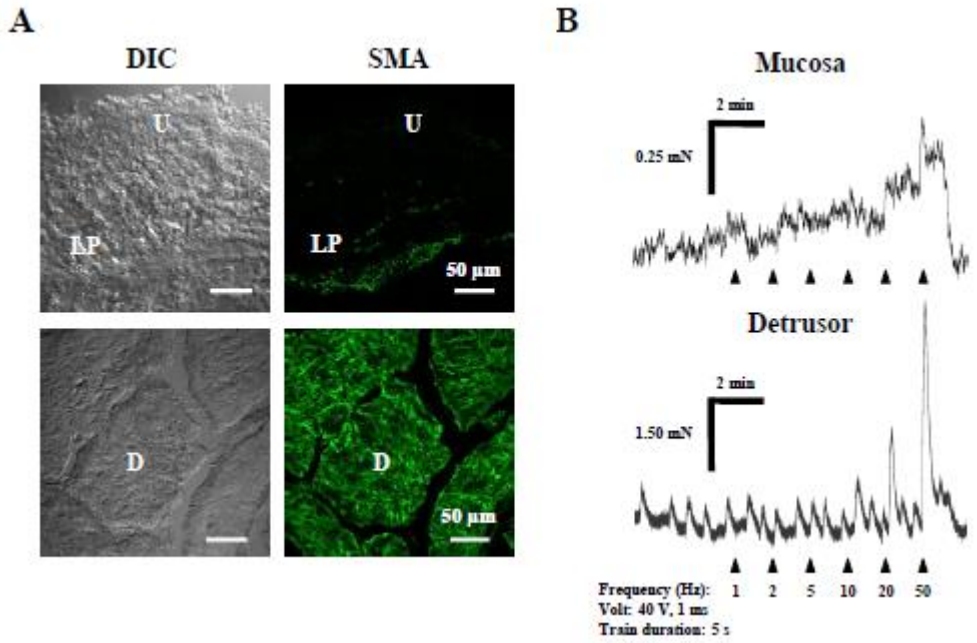


Figure 5. (A) Relative abundance of contractile elements, such as α-smooth muscle actin (SMA), in transverse sections of the human urothelium (U) with lamina propria (LP) attached compared to the detrusor (D) smooth muscle layer. Differential interference contrast (DIC) is show to facilitate delimitation of tissue boundaries. Scale bars = 50 µm. Myographic recordings shown in **(B)** were obtained in parallel using mucosal and detrusor strips from a 59-years old HBP patient, which were mounted in organ baths continuously superfused with gassed (95% O_2 and 5% CO_2) Tyrode's solution (pH 7.4); please note that tension scale bars in the two graphs are different. Despite mucosal SMA-immunoreactivity is scarce, the isolated strips from the human mucosa contract in response to electrical field stimulation (EFS, 40 V square pulses of 1 ms duration, delivered at 1-50 Hz frequency).

After a 30-min equilibration period, the preparation were loaded with 1 µM [^{3}H]choline (specific activity 2.5 µCi nmol^{-1}) during 40 min. [^{3}H]ACh release was elicited twice (S_1 and S_2) by electrical field stimulation using 200 pulses (1 ms duration, 40 V) delivered at 10 Hz frequency. Tritium content was measured by liquid scintillation spectrometry (TriCarb2900TR, Perkin Elmer, Boston, USA) (% counting efficiency: 58±2%) using 400 µL aliquots of superfusate samples collected automatically every 3 min using a fraction collector (Gilson, FC203B, France). Aliquots of these samples were immediately freeze-dried in liquid nitrogen and preserved at -80ºC for subsequent ATP quantification by bioluminescence using the luciferin-luciferase ATP kit HS II (Roche Applied Science, Indianapolis, USA) according to manufacturer's instructions (see e.g. Silva-Ramos et al., 2013; Timóteo et al., 2014; Carneiro et al., 2014). Stimulation-evoked [^{3}H]ACh and ATP release was

calculated by subtracting the basal outflow from the total outflow in the three samples collected following the stimulation period. In control conditions, transmitters release in S_2 decayed 15-20% as compared to S_1 (Figure 8, cf. Carneiro et al., 2014). Cell integrity was assessed by measuring lactate dehydrogenase (EC 1.1.1.27, intracellular enzyme) activity in superfusates.

Kinetic analysis of the extracellular ATP catabolism by HPLC

Mucosal U/LP strips were mounted in 2-mL perfusion chambers heated at 37ºC. After a 30-min equilibrium with gassed (95% O_2 and 5% CO_2) Tyrode's solution, the preparations were incubated with ATP (30 µM) (zero time). Samples of 75 µl were collected from the organ bath at different times up to 45 min for HPLC (with UV) detection) analysis (LaChrome Elite, Merck, Germany) of the variation of substrate disappearance and products formation (see e.g. Kumar et al., 2004).

Chemicals and Solutions

A317491 (5-[[[(3-phenoxyphenyl)methyl][(1S)-1,2,3,4-tetrahydro-1-naphthalenyl]amino]carbonyl]-1,2,4-benzenetricarboxylic acid sodium salt hydrate), BAPTA-AM (1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis acetoxymethyl ester), carbenoxolone, MRS2578 (N,N''-1,4-butanediylbis[N'-(3-isothiocyanatophenyl)thiourea), PSB0474 (3-(2-oxo-2-phenylethyl)-uridine-5'-diphosphate disodium salt), tetrodotoxin citrate (TTX) were from Tocris Bioscience (Bristol, UK); Choline chloride and foetal bovine serum were from Sigma (St. Louis, MO, USA); [methyl^{3}H]choline chloride (in ethanol, 85.5 Ci.mmol^{-1}) was from Perkin Elmer (Boston, USA). MRS2578 and A317491 were dissolved in dimethylsulphoxide (DMSO) stock solution. Other drugs were prepared in Tyrode's solution. No statistical differences between control experiments, made in the absence or in the presence of DMSO at the maximal concentrations used (0.5% v.v^{-1}), were observed.

Immunofluorescence Staining and Confocal

Microscopy Observation

Mucosal U/LP strips isolated from human bladder samples as described were stretched and fixed in periodate-lysine-paraformaldehyde solution for 16 hours at 4ºC. Sections (16 µM) were incubated with selected primary antibodies (see Table 7).

Table 7. Primary and secondary antibodies used in immunohistochemistry experiments.

Antigen	Code	Host	Dilution	Supplier
Primary antibodies:				
$P2Y_6$	APR-011	Rabbit	1:100	Alomone Labs, Jerusalem, Israel
P2X2 (H-116)	SC-25693	Rabbit	1:50	Santa Cruz Biotechnology, Dallas, TX
P2X3 (R-19)	SC-31492	Goat	1:25	Santa Cruz Biotechnology
ChAT	AB144P	Goat	1:750	Chemicon®
CK17	MAB1677	Mouse	1:150	Chemicon
Vimentin	M0725	Mouse	1:150	Dako, Glostrup, Denmark
PGP 9.5	7863-1004	Mouse	1:500	Serotec®
α-Smooth muscle actin-fluorescein isothiocyanate	F3777	Mouse	1:300	Sigma®
Secondary antibodies:		Donkey	1:1000	Molecular Probes®
Alexa Fluor® 488 anti-mouse	A-2120			
Alexa Fluor 488 anti-rabbit	A-21206			
Alexa Fluor 633 anti-goat	A-21082			
Alexa Fluor 680 anti-rabbit	A-10043			

Antibodies were diluted in incubation buffer (fetal bovine serum 5%, serum albumin 1% and Triton X-100 0.3% in phosphate buffered saline) ate 4ºC for 16 hours. After washout, incubation with secondary antibodies was performed in the dark for 2 hours at room temperature (see Table 7) (Timóteo et al., 2014; Carneiro et al., 2014). Observation were made and analyzed with a FluoView™ FV1000 laser scanning confocal microscope. Collagen autofluorescence in the lamina propria was subtracted from specific immunofluorescence signals in all confocal micrographs (Figure 6).

Pre-adsorption tests with an excess of corresponding antigen peptide sequences were performed to validate the specificity of P2X2, P2X3 and $P2Y_6$ antibodies (Figure 7).

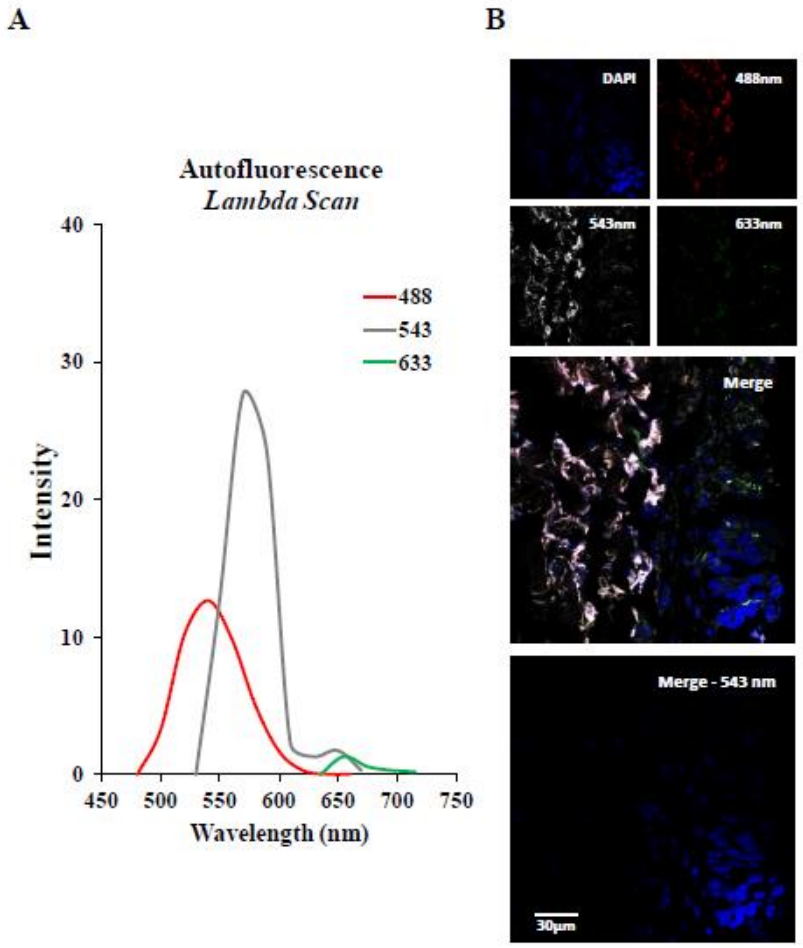


Figure 6. Method used to reduce interference of collagen autofluorescence in confocal microscopy images of mucosal slices of the human bladder. In panel **(A)**, shown are the results of lambda scan performed using 488, 543 and 633 nm excitation laser lines. Spectral emission of collagen autofluorescence was maximal in the lamina propria within the 550-625 nm range when the 543 nm excitation laser was used. The example in panel **(B)**, shows the procedure used to subtract collagen autofluorescence (emission in the 543 nm laser channel) from specific immunofluorescence signals obtained with 488 and 633 nm excitation lasers; this negative control, containing only anti-rabbit Alexa Fluor 488 and anti-goat Alexa Fluor 633 secondary antibodies (see Table 7), was counterstained with DAPI (405) to facilitate microscopic observations. The methodology exemplified here for a control human mucosal U/LP sample was applied to all confocal micrographs in this study, including those from BPH patients.

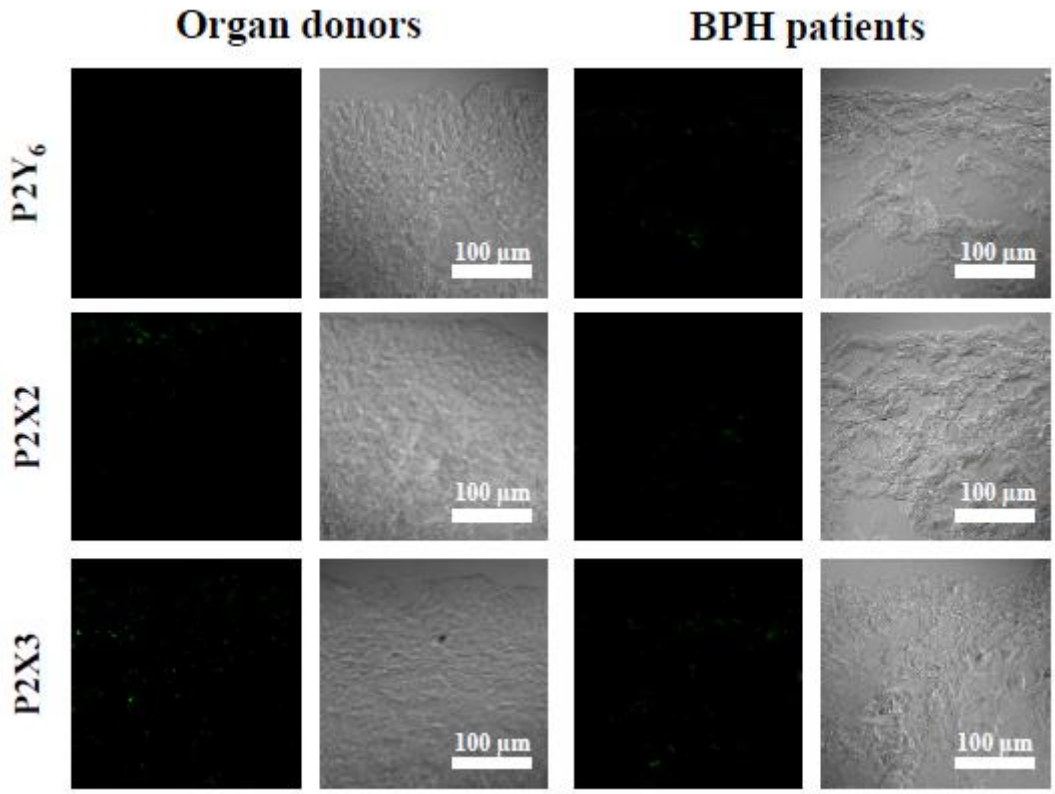


Figure 7. To test for the specificity of P2X2, P2X3 and $P2Y_6$ receptor antibodies some sections were processed with primary antibodies pre-adsorbed with 10-fold molar excess of the corresponding antigen peptide sequences, overnight at 4ºC. Sections were then processed as described in Methods with the pre-absorbed antiserum and with the normal antiserum, in parallel. During documentation of purinoceptor pre-absorption controls, settings on the confocal microscope were adjusted appropriately to show immunoreactivity for sections that were processed normally (no pre-absorption) (see e.g. figure 12) and these settings were maintained when documenting pre-absorption controls to minimize biases during capture and printing of digital images. Note that antigen pre-adsorption with the specific peptide sequences totally prevented immunofluorescence signals detection. Scale bars = 100 µm.

Data Presentation and Statistical Analysis

Results are expressed as the mean ± SEM in the number of individuals used for a particular set of experiments. Statistical data analysis was performed with GraphPad Prism®, version 6.04 for Windows®. The impaired Student t-test with the Welch correction was used for statistical analysis with 2-tailed $P<0.05$ considered significantly different.

RESULTS

P2Y$_6$ and P2X3 (and/or P2X2/3) Receptors Cooperated to Increase Nonneuronal ATP and [^{3}H]ACh Release from Control Mucosal U/LP Strips

Electrical stimulation of mucosal U/LP strips increased ATP and [^{3}H]ACh outflow (Figure 8). Stimulation induced release was not affected by blocking nerve action potentials with TTX (1 μM), suggesting that ATP and [^{3}H]ACh are mostly release from nonneuronal stores (Yoshida et al., 2010; Sadananda et al., 2009). The negligible (less than 8.0 mU/ml) activity of lactate dehydrogenase in bath samples collected before and after electrical field stimulation of the bladder mucosa in an indicator of cell integrity. Electrical stimulation of mucosal strips of the human bladder increased tissue tension above that of spontaneous phasic activity in a frequency dependent manner (Figure 5) (Moro and Chess-Williams, 2012). The UDP analogue and selective P2Y$_6$ receptor agonist PSB0474 (100 nM with 15-minute incubation) significantly increased ATP and [^{3}H]ACh release from the stimulated mucosa of control bladder samples (P< 0.05, Figure 8, A and B). Pretreatment with the selective and irreversible P2Y$_6$ receptor antagonist MRS2578 (50 nM in S_1 and S_2), prevented the facilitatory effect of PS0474 (100 nM) (Figure 8, C). Urinary ATP can cause more ATP to be released by urothelial cells and this vicious cycle may occur through the activation of P2X3 and/or P2X2/3 receptors (Sun and Chai, 2006). Blockade of positive feedback operated via P2X3 and/or P2X2/3 receptors with A317491 (100 nM in S_1 and S_2) attenuated PSB0474 (100 nM) induced mucosal release of ATP and [^{3}H]ACh (Figure 8, C).

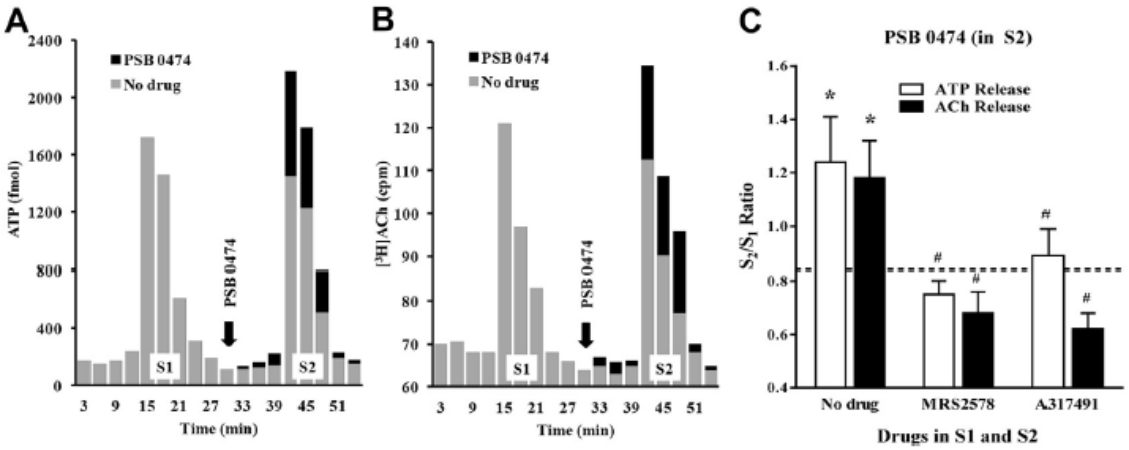


Figure 8. Facilitatory effect of selective $P2Y_6$ receptor agonist PSB0474 on ATP and [^{3}H]ACh release from stimulated mucosal strips of control human bladders. PSB0474 (100 nM) was added 15 minutes before S_2 (arrow). $P2Y_6$ antagonist MRS2578 (50 nM) and P2X3 and/or P2X2/3 antagonist A317491 (100 nM) were added to incubation medium ate beginning of release period (time zero) and were present throughout assay, including S_1 and S_2. Graphs show outflow of ATP **(A)** and [^{3}H]ACh **(B)** from human mucosal U/LP strips in typical experiments. Change in ratio between evoked ATP and [^{3}H]ACh release during two stimulation periods (S_2/S_1) relative to that in control conditions in absence of test drugs (dashed horizontal line) was considered measure of effect of PSB0474 **(C)**. In control conditions S_2/S_1 was 0.83 ± 0.05 and 0.83 ± 0.06, respectively. Bars represent SEM of 4 to 6 individuals. Dashed horizontal line indicates S_2/S_1. Asterisk indicates significantly different vs control S_2/S_1 (unpaired Student t test with Welch correction $P< 0.05$). Pound sign indicates significantly different vs PSB0474 alone (unpaired Student t test with Welch correction $P< 0.05$).

BPH Patients

Mucosa Released Higher Amounts of ATP via Pannexin-1 Hemichannels with Tonic Control by $P2Y_6$ Receptor

Figure 9 shows that in vitro mucosal U/LP strips from BPH patients release more ATP than strips from the control group ($P< 0.05$). The relative proportion of ATP compared to [^{3}H]ACh release per mg wet tissue in matched samples of 6 patients with BPH (mean 35.63 ± 7.91) and 5 control men (mean 6.81 ± 1.47) indicates that this ration was fivefold higher in the mucosa of obstructed bladders (Kumar et al., 2004). HPLC experiments to study the kinetics of extracellular ATP catabolism showed that the half degradation time of ATP (30 µM) by ectonucleotidases increased from a mean of 35 ± 3 minutes in 4 control men to a mean of 61 ± 16 minutes in 4 patients with BPH ($P< 0.05$). This may also contribute to keep high nucleotide levels in the bladder mucosa. The increase in the purinergic component originating from the mucosa of BPH patients is under tonic control of the UDP sensitive $P2Y_6$ receptor because it was down-regulated by MRS2578 (50 nM in S_1 and S_2). On its own the $P2Y_6$ receptor antagonist decrease ($P< 0.05$) evoked a ATP release per mg wet tissue from a mean of 392 ± 179 fmol/mg in 6 in BPH patients to 48 ± 6 fmol/mg in 4 and from 109 ± 25 fmol/mg in 5 controls to 62 ± 11 fmol/mg in 4 without much affecting the outflow of [^{3}H]ACh ($P > 0.05$).

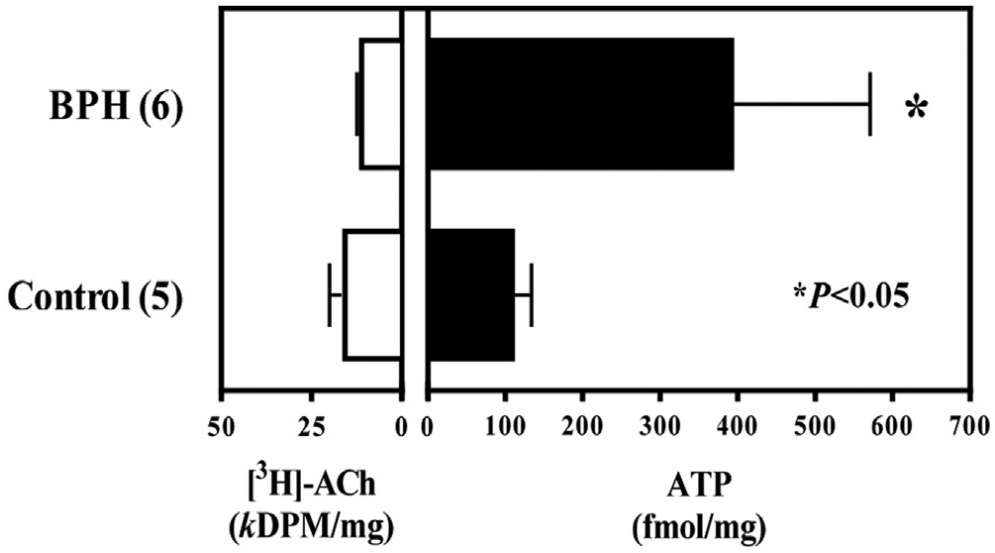


Figure 9. Amount of ATP and [^{3}H]ACh release from stimulated mucosal U/LP strips of patients means of 69 ± 4 years old with outflow obstruction due to BPH and control organ donors mean of 59 ± 4 years old. DPM, disintegrations per minute. X axis represents ATP and [^{3}H]ACh content in samples collected during S_1 in absence of test drugs. Bars represent SEM of matched mucosal bladder from 5 controls and 6 BPH patients. Asterisk indicates significantly different vs control (unpaired Student t test with Welch correction $P < 0.05$).

The PSB0474 (100 nM) induced surplus ATP release from TTX insensitive nonneuronal mucosal stores was not modified by removing Ca^{2+} and supplementing incubation medium with the fast intracellular Ca^{2+} chelator BAPTA-AM (5 μM in S_1 and S_2). Mean S_2/S_1 ratios were 1.24 ± 0.07 in 6 preparations and 1.17 ± 0.06 in 5, respectively. Conversely carbenoxolone applied in S_1 and S_2 at a concentration (10 μM) that inhibited pannexin-1 containing hemichannels in the rat bladder (Timóteo et al., 2014) virtually abolished stimulation induced ATP outflow from human mucosal strips without modifying the release of [^{3}H]ACh in 4 preparations (data not shown).

Loss of Cooperation between $P2Y_6$ and P2X3 or P2X2/3 Receptors in Bladder Mucosa

Activation of $P2Y_6$ receptor with PSB0474 (100 nM with 15- minute incubation) increased the evoked release of ATP and [^{3}H]ACh to a similar extent from the bladder mucosa of controls and BPH patients (Figure 10). As in control men, selective blockade of $P2Y_6$ receptor with MRS2578 (50 nM in S_1 and S_2) also prevented PSB0474 (100 nM) induced facilitation of ATP and [^{3}H]ACh release from mucosal strips of BPH patients (Figure 10). This was not verified regarding

cooperation between $P2Y_6$ and P2X3 (or P2X2/3) receptors, given that the P2X3 (and/or P2X2/3) receptor antagonist A317491 (100 nM in S_1 and S_2) did not attenuate PSB0474 (100 nM) facilitation of evoked [^{3}H]ACh release (Figure 11).

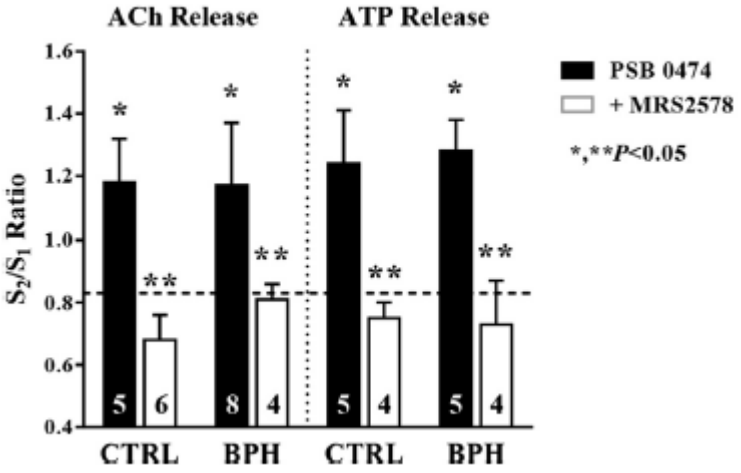


Figure 10. Activation of $P2Y_6$ receptor with PSB0474 facilitated similar ATP and [^{3}H]ACh outflow in mucosal U/LP strips from patients mean of 70 ± 4 years old. PSB0474 (100 nM) was added 15 minutes before S_2 and selective $P2Y_6$ receptor antagonist MRS2578 (50 nM) was present throughout assay, including S_1 and S_2. Ordinates indicate changes in S_2/S_1 in presence of PSB0474 (100 nM) *vs* corresponding S_2/S_1 in control conditions (dashed horizontal line). In absence of added drugs S_2/S_1 was 0.83 ± 0.05 and 0.83 ± 0.06 for [^{3}H]ACh and ATP release in controls, and 0.84 ± 0.04 and 0.86 ± 0.04, respectively, in BPH patients. Data are shown as mean ± SEM of 4 to 8 individuals. Single asterisk indicates significantly different *vs* control (unpaired Student t test with Welch correction $P < 0.05$). Double asterisks indicate significantly different *vs* PSB0474 alone (unpaired Student t test with Welch correction $P < 0.05$).

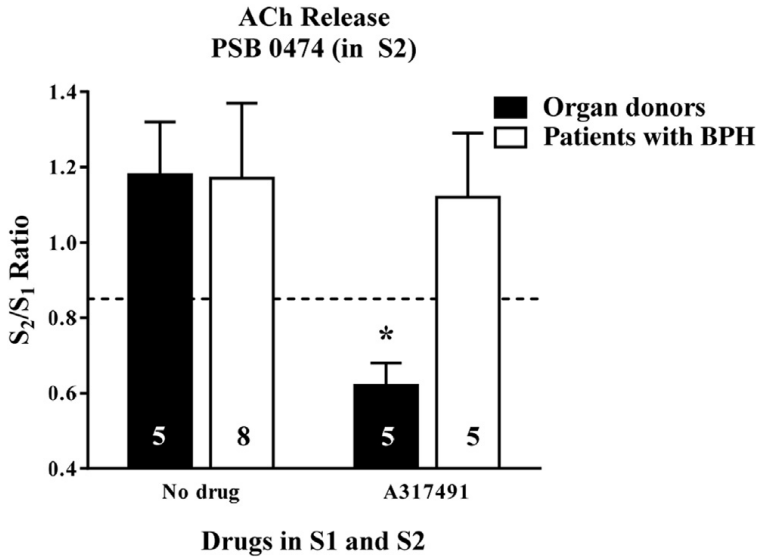


Figure 11. Blockade of P2X3 and/or P2X2/3 receptors with A317491 attenuated facilitation of [^{3}H]ACh release from stimulated U/L strips induced by PSB0474 in control organ donors mean of 59 ± 8 years old but not in patients with BHP mean of 71 ± 4 years old. PSB0474 (100 nM) was added 15 minutes before S_2 and P2X3 and/or P2X2/3 antagonist A317491 (100 nM) was present throughout assay, including S_1 and S_2. Ordinates indicate changes in S_2/S_1 in presence of PSB0474 (100 nM) compared to corresponding S_2/S_1 in control conditions (dashed horizontal line). In absence of added drugs S_2/S_1 was 0.83 ± 0.05 and 0.83 ± 0.06 for [^{3}H]ACh and ATP release in controls, and 0.84 ± 0.04 and 0.86 ± 0.04, respectively, in BPH patients. Data are shown as mean ± SEM of 5 to 8 individuals. Asterisk indicates significantly different *vs* PSB0474 alone (unpaired Student t test with Welch correction $P < 0.05$).

Changes in P2 Purinoceptor and ChAT Immunostaining Patterns in Mucosal U/LP Strips

The presence of $P2Y_6$, P2X2, P2X3 receptors and ChAT (the enzyme that syntheses ACh) in human urothelium was demonstrated by immunofluorescence confocal microscopy (Figure 12). P2X2, P2X3 and $P2Y_6$ receptors were also observed in the suburothelial layer. The presence of ChAT and, thus, the ability to synthesize and release ACh decreased significantly in the urothelium of BPH patients compared to healthy controls (Figure 9). Figure 12 shows that immunoreactivity against P2X2 and P2X3 receptors almost disappeared from the urothelium of BHP patients. In the suburothelial layer P2X2 receptor is predominantly localized in blood vessels and vimentin positive (myo)fibroblasts while P2X3 receptors stained PGP 9.5 positive suburothelial nerve fibers (Figures 12 and 13, A and B). In contrast to the partial loss of ionotropic P2X receptor staining, immunoreactivity against $P2Y_6$ receptor remained almost unaltered in the urothelium of BHP patients compared to control men (Figure 12). However, labeled cells were more sparse, denoting increased urothelial permeability (Mirone et al., 2007). $P2Y_6$ receptor was localized throughout the urothelial layer, including the more superficial umbrella cells stained with CK17 (Figure 13, C). Suburothelial $P2Y_6$ receptors stained more intensely in BHP patients (Figure 12).

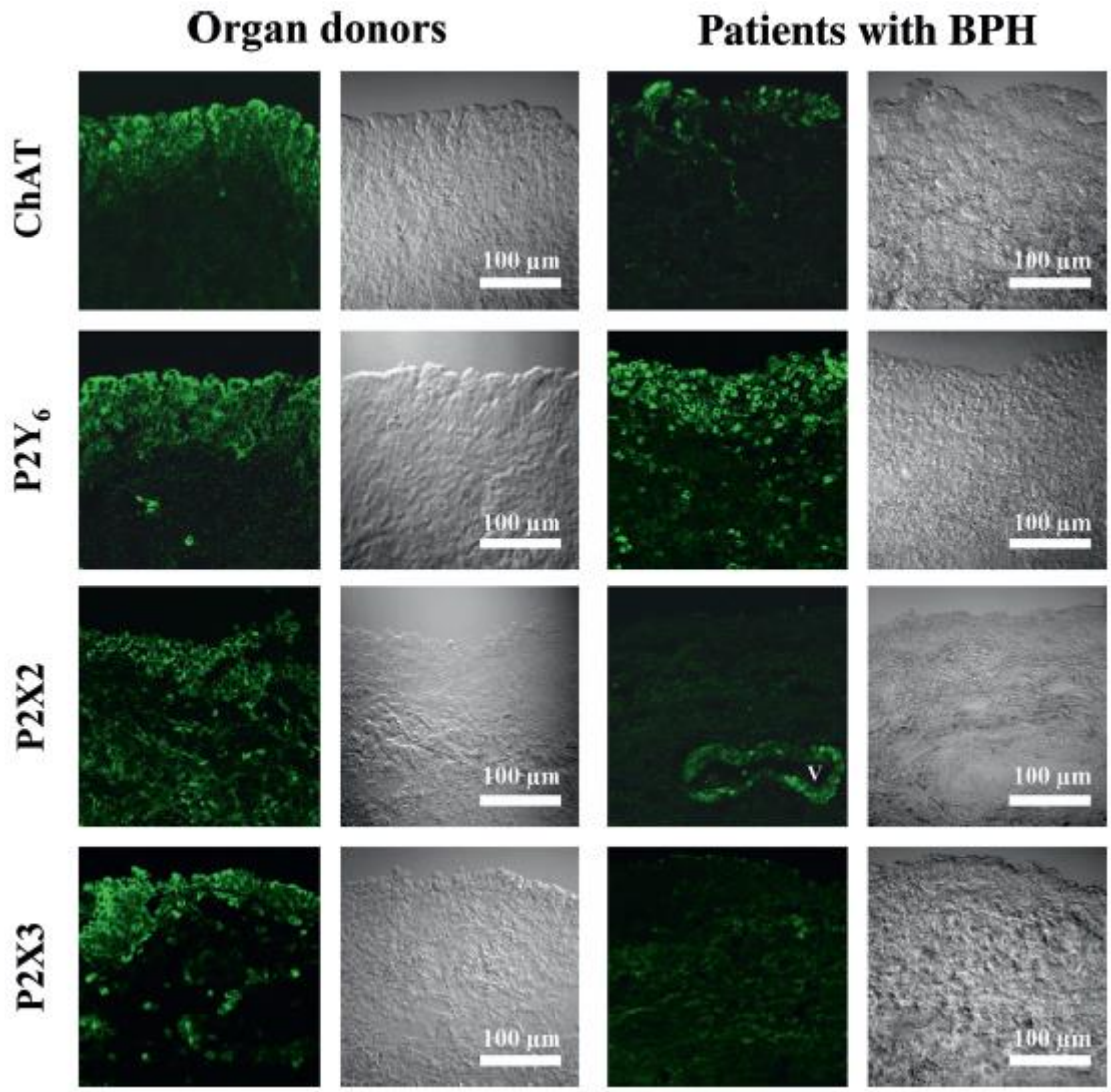


Figure 12. Color confocal micrographs show immunoreactivity against ChAT and P2 purinoceptors (green areas), namely $P2Y_6$, P2X2 and P2X3, in transverse U/L sections from control organ donors and patients with outflow obstruction due to BPH. Black and white images are differential interference contrast images. Data represent 5 individuals per group, including organ donors (ChAT and $P2Y_6$ section H79OD, and P2X2 and P2X3 section H75OD) and BPH patients (ChAT and P2X2 section H24BPH, and $P2Y_6$ and P2X3 section H77BPH). V. blood vessel. Scale bars indicate 100 µm.

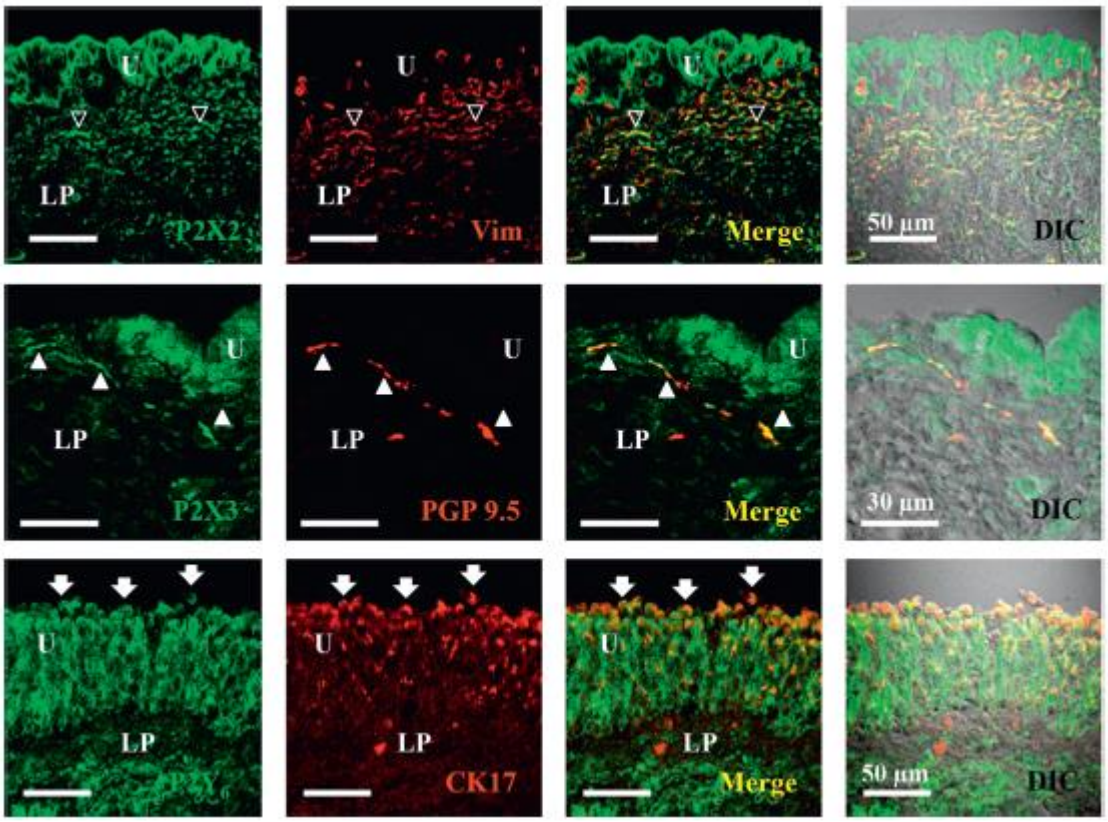


Figure 13. Co-localization experiments of P2X2, P2X3 and $P2Y_6$ receptors with type specific cell markers in human bladder mucosal urothelium (U) attached to lamina propria (LP). Urothelial cells, fibroblasts and suburothelial nerve fibers (red areas) were stained with CK17, vimentin (Vim) and PGP 9.5, respectively. To facilitate visualization of small nerve fibers stained with PGP 9.5 images correspond to higher magnification intensity projections over Z axis of 6 confocal stacks. P2X2 receptors (green areas) co-localized with vimentin positive fibroblast cells (open triangles) while PGP 9.5 stained suburothelial nerve fibers expressed P2X3 receptors (solid triangles). Co-localization (yellow areas) of $P2Y_6$ receptor (green areas) and CK17 (red areas) was observed throughout urothelium, including most superficial umbrella cells (arrows). Differential interference contrast (DIC) images are shown to facilitate delimitation of tissue boundaries. Scale bars indicate 30 or 50 µm.

DISCUSSION

To our knowledge immunofluorescence confocal microscopy data showed for the first time that the UDP sensitive $P2Y_6$ receptor is abundantly expressed in human urothelium, including the highly differentiated superficial umbrella cells. Besides its presence in the urothelial layer, the expression of $P2Y_6$ receptors in suburothelial myofibroblasts and detrusor smooth muscle fibers has also been reported (Timóteo

et al., 2014; Carneiro et al., 2014; Yu et al., 2013; Sui et al., 2006). More importantly we found that activation of $P2Y_6$ receptor facilitated the release of ATP and ACh from nonneuronal urothelial sources, thus, contributing to initiation and local propagation of signals from the human urothelium to underlying bladder layers, namely suburothelial nerve fibers, myofibroblasts and detrusor smooth muscle, which are endowed with P2 and cholinergic receptors (Winder et al., 2014; Fry et al., 2012). Cooperation between metabotropic $P2Y_6$, and ionotropic P2X3 and/or P2X2/3 receptors in the human urothelium with lamina propria contributes to enhance ATP and ACh release. These findings fully agree with data from our group demonstrating that urothelial $P2Y_6$ receptors act synergistically with P2X3 receptors and both have important roles in the mechanosensory purinergic pathway commanding the micturition reflex in anesthetized rat without much affecting the motor drive during bladder voiding (Carneiro et al., 2014). The expression and function of $P2Y_6$ receptor was conserved in the urothelial layer of BPH patients while we noted a relative intensification of $P2Y_6$ receptor staining in suburothelial myofibroblasts compared to that in control men. This pattern contrasted with the loss of P2X2 and P2X3 immunoreactivity from the urothelium of BPH patients, comparable to that observed in other pathological conditions of the human bladder (Ruggieri, 2006). These findings may explain why the potent and selective P2X3 and P2X2/3 receptor antagonist A317491 became inactive upon transmitter release induced by $P2Y_6$ receptors activation in BPH patients. P2X3 and the heterodimer P2X2/3 are fast desensitizing ionotropic receptors that are activated almost exclusively by ATP. Decreased activity of ectonucleotidases has been shown in overactive bladders, leading to increased smooth muscle responses to ATP (Harvey et al., 2002). Thus, it seems possible that extracellular ATP accumulation as in this study might contribute to the loss of P2X2 and P2X3 receptor staining in these patients.

Bladder wall thickness due to smooth muscle hypertrophy and connective tissue infiltration, accompanied by autonomic denervation of the detrusor, has been found in decompensated bladders secondary to BPH (Levin et al., 2000). However, less is known regarding morphological changes operating in the mucosa of these patients, which may be important to explain the pathophysiology of obstructed bladders. This study shows that gross morphological alterations hardly sustained immunohistochemical differences in the bladder mucosa of controls and BPH patients, prompting more relevant modifications in gene expression and transcription

to explain mechanically induced deregulation of the synthesis of many proteins such as receptors, ionic channels and enzymes (Mirone et al., 2007).

Notwithstanding the lack of the positive feedback mechanism operated by released ATP via urothelial P2X3 and/or P2X2/3 receptors in BPH patients, the magnitude of the $P2Y_6$ mediated facilitation of ATP and [^{3}H]ACh release from mucosal U/LP strips was essentially conserved. This paradox can be explained by considering 1) up-regulating of $P2Y_6$ receptors in the suburothelial layer, probably in myofibroblasts, and 2) prolongation of ATP half-life in obstructed bladders. Thus, the significant increase in the ATP/ [^{3}H]ACh release ratio verified in the mucosa of BPH patients can be partially attributable to deficits in the expression and/or activity of ATP metabolizing enzymes, namely NTPDases, along with partial loss of ChAT, which together with the mitochondrial enzyme carnitine acetyltransferase are responsible for ACh synthesis in the urothelium (Lips et al., 2007). Our data suggest that bladder outlet obstruction counteracted age related increases in nonneuronal ACh release (Yoshida et al., 2004) while favoring mucosal ATP accumulation (Figure 9) because no significant changes were observed in the ACh content of mucosal strips from BPH patients compared to the younger control group. Whether this impacts antimuscarinic drug resistance to treat bladder overactivity in BPH patients remains to be elucidated.

While huge amounts of extracellular ATP may leak from injured cells, the mechanism of ATP release from intact urothelial cells is still a matter of debate. In this study ATP release from mucosal U/LP strips was not due to cell damage because we detected no significant changes in the activity of the intracellular enzyme lactate dehydrogenase in incubation fluid. We also found that the stimulation evoked ATP and [^{3}H]ACh release from human mucosal strips originated from TTX insensitive nonneuronal stores and was Ca^{2+} independent, thus, excluding a major contribution of vesicular exocytosis. Nonvesicular release of ACh from urothelial cells has been shown to occur via the organic cation transporters OCT1 and OCT3 (Winder et al., 2014; Lips et al., 2007). Stretch induced ATP release from the urothelium is mainly attributable to vesicular membrane traffic in umbrella cells (Wang et al., 2005) but nonvesicular mechanism of ATP outflow may also exist under various pathophysiological conditions. A significant role of pannexin-1 containing hemichannels on $P2Y_6$ receptor mediated ATP release to control bladder urodynamics has been demonstrated in the anaesthetized rat (Timóteo et al., 2014).

Likewise we report that the pannexin-1 inhibitor carbenoxolone significantly attenuated stimulation evoked and PSB0474 induced ATP release facilitation. Although carbenoxolone may also block connexin containing hemichannels, their involvement in ATP release under the current conditions is less plausible. This is because, unlike pannexin-1, connexin hemichannels close at normal millimolar Ca^{2+} (eg 1.8 mM $CaCl_2$ in Tyrode solution) and open under Ca^{2+} depletion conditions (Fasciani et al., 2013). This supports our hypothesis that pannexin-1 containing hemichannels may have a relevant role in the outflow of ATP from the human bladder mucosa.

The observation that selective blockade of the UDP sensitive $P2Y_6$ receptor with MRS2578 decreased ATP outflow from mucosal strips suggests that UTP or UDP is endogenously released. Considerable amounts of UTP and UDP were detected along with ATP and ADP in urine specimens of asymptomatic controls and patients with overactive bladder with a trend toward higher urinary levels of ATP and UDP in the patient group (Contreras-Sanz et al., 2012). Inhibition of ATP release by MRS2578 was more pronounced in mucosal strips from BPH patients than from control men while no changes were found regarding [^{3}H]ACh outflow in both groups. Therefore, one may assume that uracil nucleotides, in particular UDP, are most likely to affect (via the $P2Y_6$ receptor) ATP outflow from stimulated mucosal strips of BPH patients.

CONCLUSIONS

Data suggest that down modulation of transmitters release from the bladder sensory system with selective $P2Y_6$ receptor antagonists may be a novel therapeutic strategy to control persistent storage symptoms in patients with BOO. Further studies interplay between various P2 purinoceptors to control nonneuronal reflexes in the human bladder mucosa.

ACKNOWLEDGEMENTS

This research was partially supported by Fundação para a Ciência e a Tecnologia (FCT, FEDER funding, projects PTDC/SAU-OSM/104369/2008,

REEQ/1168/SAU/2005, REEQ/1264/SAU/2005 and PEst-OE/SAU/UI0215/2014), Associação Portuguesa de Urologia (APU) and University of Porto/Caixa Geral de Depósitos (Investigação Científica na Pré-Graduação). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. Isabel Silva was in receipt of a PhD Studentship from FCT (SFRH/BD/88855/2012). The authors wish to thank Mrs. Helena Costa e Silva and Belmira Silva for their valuable technical assistance.

PAPER 2

Manuscript in preparation

Balance between excitatory P2X2/3 and inhibitory $P2Y_{12}$ receptors in men with prostatic bladder outlet obstruction to control cholinergic nerve activity

Isabel Silva[1,2], Fátima Ferreirinha[1,2], M. Teresa Magalhães-Cardoso[1,2], Miguel Silva-Ramos[1,2,3], Julie Pelletier[4], Jean Sévigny[4,5] & Paulo Correia-de-Sá*[1,2]

[1]Laboratório de Farmacologia e Neurobiologia, [2]Center for Drug Discovery and Innovative Medicines (MedInUP), Instituto de Ciências Biomédicas Abel Salazar (ICBAS), Universidade do Porto, [3]Serviço Urologia, Centro Hospitalar do Porto (CHP), Porto, Portugal, [4]Centre de Recherche du CHU de Québec, CHUL, [5]Centre de Recherche du CHU de Québec, CHUL/Département de Microbiologie-Infectiologie et d'Immunolohie, Faculté de Médicine, Université Laval, Québec, QC, Canada.

* Corresponding author: farmacol@icbas.up.pt

Contribution of authors: PCS supervised the project, designed the experiments, and analyzed data. IS, and PCS wrote the paper. IS and MTMC performed and analyzed data from enzymatic kinetic experiments by HPLC. IS performed and analyzed data from acetylcholine release experiments. IS and FF performed immunofluorescence staining and confocal microscopy observations. MSR recruited the patients and collected the bladder samples. JP and JS produced the antibodies against ecto-nucleotidases. IS, FF, MTMC, MSR, JP, JS and PCS interpreted data, discussed the clinical implications, and commented on the manuscript at all stages.

ABSTRACT

Purines have important roles in the regulation of human bladder function. Purinergic signaling increases significantly in patients with lower urinary tract symptoms (LUTS), such as overactive bladder (OAB) and bladder outflow obstruction (BOO) due benign prostatic hyperplasia (BPH). Our group demonstrated that uroepithelial cells and cholinergic nerves from men with BOO due to BPH release 3-5 fold more ATP than control individuals. It is, therefore, tempting to admit that the purinergic signaling cascade may be a novel therapeutic target to control bladder overactivity when anticholinergic drugs and β_3-adrenoceptor agonists fail or are contraindicated. ATP released from the urothelium during bladder filling is responsible for the sensory input to the central nervous system by activating ionotropic P2X3 and/or P2X2/3 receptors on suburothelial nerve afferents. ATP is

also involved in pathological bladder contractions through the activation of P2X1 receptors on smooth muscle fibers, but so far no attempts have been made to show a neuromodulatory role of the nucleotide in the human bladder. ATP-induced bladder excitability may be cut-short by membrane-bound NTPDases, which sequentially hydrolyze ATP into ADP, AMP and adenosine. Like adenosine, ADP also restrains cholinergic neuromuscular transmission in the bladder via inhibitory P2Y receptors, which subtype has not yet been identified. In this study, we aimed at investigating the extracellular catabolism of ATP by subtype-selective NTPDases in the detrusor of men with BOO due to BPH, as well as the interplay between ATP-sensitive ionotropic P2X and ADP-activated metabotropic P2Y receptor subtypes to control hyperactivity of cholinergic nerves in the pathological human bladder. The kinetics of the extracellular catabolism of ATP and ADP is 1.4 and 1.9 times slower in the detrusor of BPH patients than in control individuals. This difference was mainly attributed to decreases in NTPDase1/CD39 (ATP diphosphohydrolase) activity converting both ATP and ADP directly into AMP, with subsequent dephosphorylation to adenosine by ecto-5'-nucleotidase/CD73 on smooth muscle fibres. Only minor changes were detected in the expression of NTPDase2 (ATPase) bound to cholinergic nerve fibres, which might favour transient ADP accumulation at the neuromuscular synapse. Differential inhibition of NTPDase1 and 2 respectively with ARL67156 (100 µM) and POM-1 (100 µM) decreased evoked [^{3}H]ACh release by 13±8% (n=5) and 24±6% (n=5) in BOO patients, but the two compounds were about equipotent (~30%) on evoked transmitter release in control individuals. While the P2X3 receptor antagonist, A317491 (100 nM), decreased [^{3}H]ACh release by 20±2% (n=4) and 24±6% (n=5) in control and BOO patients, respectively, the P2X2/3 receptor blocker, TNP-ATP (10 nM), was more effective in BOO patients (43±16%, n=4) than in controls (27±9%, n=4). Selective antagonism of the ADP-sensitive $P2Y_{12}$ receptor with AR-C66096 (100 nM), but not of $P2Y_1$ and $P2Y_{13}$ receptors with MRS2179 (300 nM) and MRS2211 (10 µM), increased [^{3}H]ACh release by 23±9% (n=5). Data suggest that transient accumulation of ADP leading to inhibition of cholinergic neurotransmission via $P2Y_{12}$ receptors may partially counteract hyperactivity of cholinergic nerves operated by surplus ATP through ionotropic P2X2/3 receptors activation in men with BPH.

Keywords: human detrusor bladder, ecto-NTPDases, ADP-sensitive receptors, P2X3 receptors, P2X2/3 receptors.

INTRODUCTION

ATP is involved in the mechanosensory function of most hollow organs (Burnstock, 2007). In the urinary bladder, ATP released upon distension of the urothelium during bladder filling activates the P2X3 receptor on suburothelial sensory nerve fibers to initiate the micturition reflex (Calvert et al., 2008). Activation of heteromeric P2X2/3 receptors localized on lumbosacral and splanchnic nerves may also participate in bladder sensation to stretch, pressure and irritation (Ford, 2012). Mice lacking P2X2, P2X3, or both receptors have reduced bladder reflexes and increased bladder volume threshold (Ford, 2012). Selective P2X3 receptor antagonists, including A317491, reduce bladder hyperactivity in experimental models (Lu et al., 2007; Brederson and Jarvis, 2008), thus supporting the theory that blockade of P2X3 receptors may be therapeutically useful to control overactive bladder (OAB), interstitial cystitis and bladder hyperactivity due to bladder outlet obstruction (BOO) (Burnstock, 2007; Ford, 2012).

Localization of ATP-sensitive P2 receptors subtypes in the human urinary bladder is not fully resolved. The P2X2 receptor is expressed predominantly in vimentin-positive cells located in the suburothelium, with lower levels of expression in actin-positive smooth muscle fibers. The P2X3 receptor is also expressed in vimentin-positive cells in the suburothelium and in those surrounding smooth muscle fibers (Svennersten et al., 2015). Both P2X2 and P2X3 receptors are also present in choline acetyltransferase-positive urothelial cells, but the P2X2 and P2X3 immunoreactivity decreases in the urothelium of patients with BOO due BPH; this situation is contrary to the increase in UDP-sensitive $P2Y_6$ receptor staining in the same cells (Silva et al., 2015). Coincidently or not, activation of the $P2Y_6$ receptor, but not P2X2 and P2X3 receptors facilitates the release of ATP and ACh from the human urothelium and this effect is particularly evident in patients with BOO due to BPH (Silva et al., 2015).

Our group demonstrated that women with overactive bladder (Silva-Ramos et al., 2013) and men with BOO due to BPH (Silva-Ramos et al., 2016a) have increased

levels of urinary ATP. These findings led us to hypothesize that the urinary ATP may be a valuable biomarker of detrusor competence, which may be useful to distinguish low urinary flow rates due to BOO from detrusor underactivity (Silva-Ramos et al., 2016a). Patients with BOO also exhibit impaired NTPDase1/CD39 activity, the enzyme responsible for the extracellular hydrolysis of ATP directly into AMP (Silva-Ramos et al., 2015a). Reduced ATP hydrolysis due to deficient NTPDase1/CD39 activity might explain (1) urinary ATP accumulation, and (2) shortage of adenosine leading to A_1 receptor-mediated inhibition of cholinergic nerve activity in the bladder of obstructed human patients (Silva-Ramos et al., 2015a). Thus, cholinergic hyperactivity due to adenosine deficiency together with increased activation of ionotropic P2X1 receptors in detrusor smooth muscle fibers by accumulating ATP contribute to detrusor overactivity (Kennedy et al., 2007; Silva-Ramos et al., 2016b).

Despite our knowledge about the pathophysiological role of ATP and adenosine in the urinary bladder, no attempts have been made so far to investigate the role of the ATP intermediate metabolite, ADP, in the human detrusor. ADP signals are mediated preferentially by metabotropic $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptors (Communi et al., 2001; Marteau et al., 2003). Activation of the $P2Y_1$ receptor inhibits ACh release from cholinergic nerves of the rat urinary bladder (Carneiro et al., 2014). Using myographic recordings, Yu et al. (2014) showed that ADP causes smooth muscle contraction in the mouse bladder through the activation of the $P2Y_{12}$ receptor, without the participation of $P2Y_1$ and $P2Y_{13}$ receptors. The identification of the $P2Y_{12}$ receptor in smooth muscle fibers offers potentially novel treatment strategies for bladder contractility disorders using clinically available $P2Y_{12}$ receptor antagonists, such as the antithrombotic drug clopidogrel.

In this study, we aimed at investigating the extracellular catabolism of ATP and ADP by subtype-selective NTPDases in the detrusor of men with BOO due to BPH, as well as the interplay between ATP-sensitive ionotropic P2X and ADP-activated metabotropic P2Y receptor subtypes to control cholinergic neurotransmission in obstructed human bladders.

MATERIAL AND METHODS

Human Bladder Samples

Human bladder samples were collected as described by previous studies from our group (Silva-Ramos et al., 2015a; Silva et al., 2015). Urinary bladder samples were removed from the anterior wall of the bladder dome of 46 consecutive patients (mean ± SEM age of 69.87 ± 1.20 years) with bladder outlet obstruction (BOO) due benign prostate hyperplasia (BPH) who underwent transvesical prostectomy and 21 male organ donors (mean age of 49.43 ± 3.25 years) at the time of organ harvest for transplantation. Samples were immediately placed in cold (4-6ºC) mannitol transplantation solution at 400 mOsm/kg (M-400) not supplemented with ATP or adenosine (4.190 gm 100 ml $^{-1}$ mannitol, 0.205 gm 100^{-1} KH_2PO_4, 0.970 gm 100^{-1} $K_2HPO_4.3H_2O$, 0.122 gm 100 ml^{-1} KCl and 0.084 gm 100 ml^{-1} $NaHCO_3$, 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 Centro Hospitalar do Porto and Instituto de Ciências Biomédicas Abel Salazar, medical school. All patients with BPH provided signed informed consent to use the biological material. The investigation conformed to the principles outline in The Code of Ethics of the World Medical Association (Declaration of Helsinki).

[^{3}H]ACh release experiments

After dissecting out the mucosa through cleavage at the *lamina propria*, detrusor muscle strips (~1.5x5 mm) were mounted in 3 ml capacity vertical perfusion chambers heated at 37ºC. The procedures used for labeling the preparations and measuring evoked [^{3}H]ACh release were described previously (Silva-Ramos et al., 2015a; Silva et al., 2015). Briefly, the preparations were superfused with gassed (95% O_2 and 5% CO_2) Tyrode's solution (pH 7.4) containing (mM) NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1, NaH_2PO_4 0.4, $NaHCO_3$ 11.9, glucose 11.2 and choline 0.001.

After a 30-min equilibration period, cholinergic neurons were loaded with 1 μM [^{3}H]choline (specific activity 2.5 μCi nmol^{-1}) under electrical field stimulation (EFS, 1 Hz frequency, 0.5 ms pulse width, 40 V) during 40 min. Washout of the preparations was performed for 60 min by superfusion (15 mL min^{-1}) with Tyrode's solution supplemented with the choline uptake inhibitor, hemicholinium-3 (10 μM). Tritium content was measured by liquid scintillation spectrometry (TriCarb2900TR; Perkin Elmer, Boston, USA) (% counting efficiency = 58±2 %) after appropriate background subtraction, using 400 μL bath samples collected automatically every 3 min with a fraction collector (Gilson, FC203B, France). [^{3}H]ACh release was evoked by two periods of electrical field stimulation (S_1 and S_2, 200 pulses of 0.5 ms duration delivered at 10 Hz frequency). Therefore, the evoked [^{3}H]ACh release was calculated by subtracting the basal tritium outflow from the total tritium outflow during the stimulation period (see, e.g. Silva-Ramos et al., 2015a; Silva et al., 2015).

Kinetic of the extracellular catabolism of ADP by HPLC

For the kinetic experiments of the extracellular catabolism of ADP, detrusor strips without the mucosa were mounted in a 2 mL organ bath. All experiments were performed at 37ºC. Preparations were superfused with gassed (95% O_2 and 5% CO_2) Tyrode's solution. After equilibrium, the preparations were incubated with 30 μM of ADP (zero time). Samples of 75 μL were collected from the organ bath at different times up to 45 min for HPLC (with UV detection) analysis (LaChrome Elite; Merck, Germany) of the variation of substrate disappearance and product formation (Duarte-Araújo et al., 2004; Correia-de-Sá et al., 2006; Vieira et al., 2014; Silva-Ramos et al., 2015a). The stoichiometry of ADP conversion into their metabolites was kept unaltered (30 μM). Considering that the curvilinear decrease of the initial substrate with time is characteristic of first-order kinetics, the half-degradation time was estimated from polynomial fitting of linear semilogarithmic progress curves of the catabolism of adenine nucleotides for each separate experiment (see, e.g. Duarte-Araújo et al., 2004; Silva-Ramos et al., 2015a).

Immunofluorescence Staining and Confocal Microscopy Observation

Detrusor strips from human bladder samples as described were stretched and fixed in periodate-lysine-paraformaldehyde solution for 16 hours at 4ºC. Sections (16 µm) were incubated with select primary antibodies (see table 8). Antibodies were diluted in incubation buffer (fetal bovine serum 5%, serum albumin 1% and Triton X-100 0.3% in phosphate buffered saline ate 4ºC for 16 hours. After washout, incubation with secondary antibodies was performed in the dark for 2 hours at room temperature (see table 8) (Silva-Ramos et al., 2015a). Observations were made and analyzed with a FluoView™ FV1000 laser scanning confocal microscope. During documentation of detrusor sections from control and BOO patients, settings on the confocal microscope were kept unaltered to minimize bias.

Table 8. Primary and secondary antibodies used in immunohistochemistry experiments.

Antigen	Code	Host	Dilution	Supplier
Primary antibodies:				
NTPDase1	hN1-9_L(I4)	Rabbit	1:200	J Sévigny
NTPDase2	hN2-2L	Rabbit	1:200	J Sévigny
NTPDase3	nH3-1_c(I4)	Guinea-pig	1:200	J Sévigny
Ecto-5'-nucleotidase	h5'NT-2_c(I4)	Guinea-pig	1:200	J Sévigny
$P2Y_1$	APR-009	Rabbit	1:75	Alomone
$P2Y_{12}$	APR-020	Rabbit	1:50	Alomone
$P2Y_{13}$	APR-017	Rabbit	1:50	Alomone
VAChT	SAB5200240	Mouse	1:50	Sigma
Secondary antibodies:				
Alexa Fluor 488 anti-rb	A-21206	Donkey	1:1500	Mol. Probes
Alexa Fluor 568 anti-ms	A-10037	Donkey	1:1500	Mol. Probes
Dylight 649 anti-gp	706-495-148	Donkey	1:150	Jackson ImmunoR.

Drugs and solutions

Adenosine 5'-[β-thio]diphosphate trititium salt (ADPβS), pyridoxal phosphate-6-azo(benzene-2,4-disulfonic acid) tetrasodium salt hydrate (PPADS tetrasodium salt hydrate), anhydrous glycerol, choline chloride, fetal bovine, hemicholinium-3, paraformaldehyde (prills), lysine and sodium periodate were obtained from Sigma

(St. Louis, MO, USA); ADP, 5-[[[(3-Phenoxyphenye)methy][[(1s)-1,2,3,-4-tetrahydro-1-aphthalenyl]amino]carbonyl]-1,2,4-benzenetricarboxylic acid sodium salt hydrate (A317491 sodium salt hydrate), 6-N,N-Diethyl-D-β,γ- dibromomethylene ATP trisodium salt (ARL67156 trisodium salt), sodium metatungstate (POM-1), 2-(Propylthio)adenosine-5'-O-(β,γ, difluoromethylene)(triphosphate tetrasodium salt (AR-C66096 tetrasodium salt), 2',3'-0-(2,4,6-Trinitrophenyl)adenosine-5'-triphosphate tetra(triethylammonium) salt (TNP-ATP triethylammonium salt), 2-[(2-Chloro-5-nitrophenyl)azo]-5-hydroxy-6-methyl-3-[(phosphonooxy)-4-pyridinecarboxaldehyde disodium salt (MRS2211) were obtained from Tocris Bioscience (Bristol, UK); 2'-deoxy-N6-methyladenosine 3', 5'-biphosphate tetrasodium salt (MRS2179) was obtained from ABCAM (Cambridge, UK); [methyl-^{3}H] choline chloride (ethanol solution, 80.6 Cinmol^{-1}) was obtained from Perkin Elmer (Boston, USA); serum albumin, Triton X-100 were obtained from Merck (Darmstadt, Germany); The antibodies against ecto-NTPDases and ecto-5'-nucleotidase were a kind gift from J. Sévigny (Univ. Lavel, Québec, QC, Canada). PPADS was made up as 3 mmol.L^{-1}, while TNP-ATP was made up as 10 mmol.L^{-1} stock solution in distilled water. A317491 was prepared in DMSO. 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 ºC. Dilutions of these stock solutions were made daily and appropriate solvent controls were done. No statistical differences between control experiments, made in the absence or in the presence of the solvents at the maxima concentration used (0.5% v/v), were observed.

Data Presentation and Statistical Analysis

Results are expressed as the mean ± SEM in the number of individuals used for a particular set of experiments. Statistical data analysis was performed with GraphPad Prism®, version 6.04 for Windows®. The unpaired Student t-test with the Welch correction was used for statistical analysis with 2-tailed $p<0.05$ considered significantly different.

RESULTS

Expression and activity of ecto-NTPDases in the urinary bladder of control individuals and BPH patients

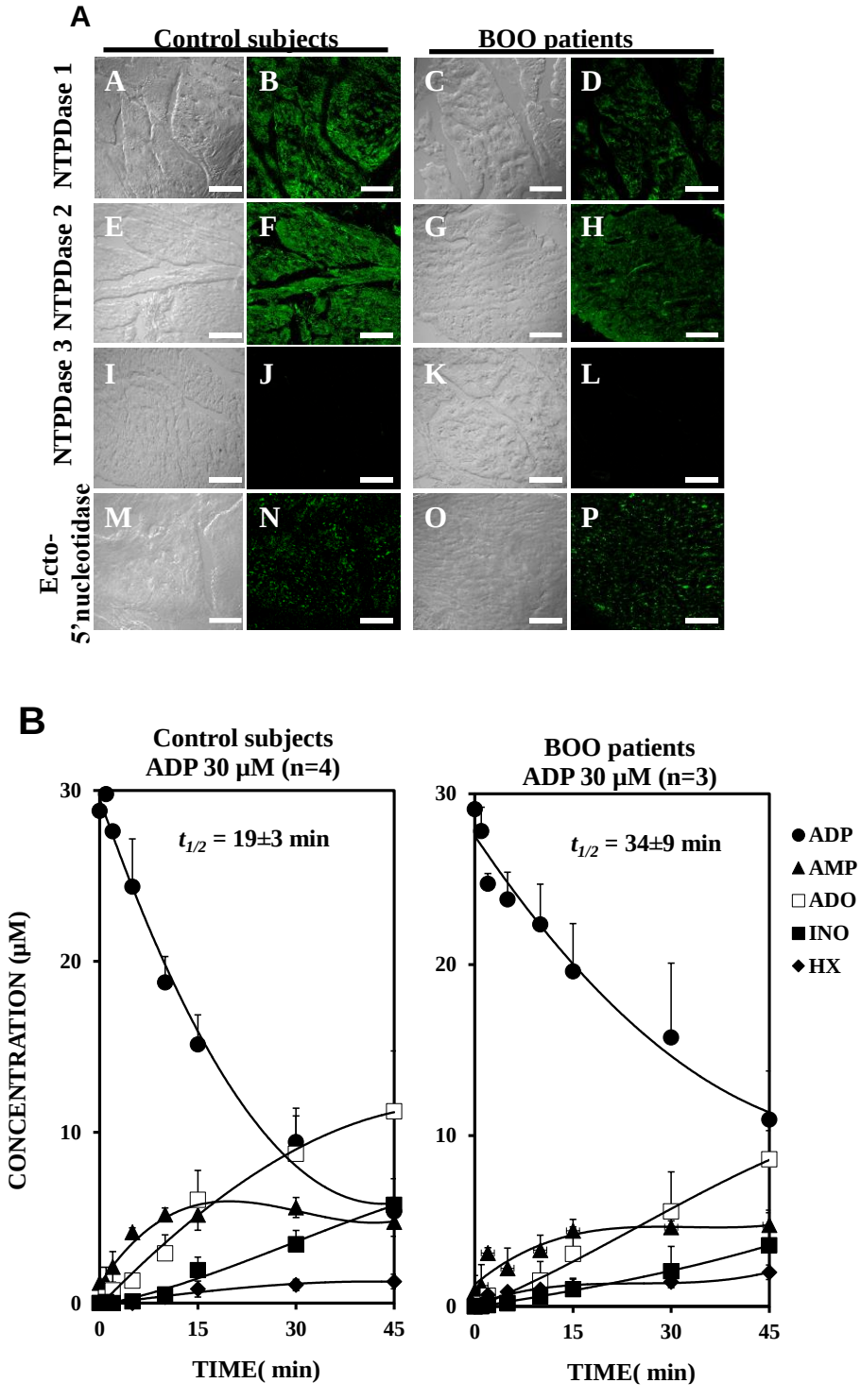


Figure 14. (A) Confocal micrographs showing the immunoreactivity against NTPDase1, 2, 3 and ecto5'-nucleotidase in transverse sections of the detrusor from organ donors (control) and BOO patients. NTPDase1, 2, 3 and ecto5'-nucleotidase immunoreactivity is shown in green. Images are representative of five individuals per group, control and BOO patients. NTPDase1, 2 (panels A-H) and ecto5'-nucleotidase (panels M-P) are the most expressed ecto-nucleotidases in the human detrusor, while immunoreactivity against NTPDase3 is not observed (panels I-L). NTPDases 1 and 2 are more evident in the detrusor of control subjects (panels B, F and N) than in BOO patients (panels D, H and P). Scale bars = 50 µm. **(B)** The kinetics of the extracellular catabolism of ADP is shower in mucosal-denuded detrusor strips from patients with outflow obstruction due to benign prostatic hyperplasia (BPH) as compared to control organ donors (control). ADP (30 µM) was added at zero time to the incubation medium. Samples were collected from the incubation fluid at the indicated times on the abscissa and analyzed by HPLC with UV detection to quantify ADP (filled circles) and its metabolites, AMP (filled triangles), adenosine (ADO, open squares), inosine (INO, filled squares) and hypoxanthine (HX, filled lozenges). Average results obtained from three to four individuals of BOO patients and control individuals, respectively; the vertical bars represent SD and are shown when they exceed the symbols in size.

Figure 14A shows the immunolocalization of ecto-NTPdases 1, 2 and 3 and ecto-5'nucleotidase/CD73 in the human detrusor from control individuals (panels B, F, J, N) and patients with bladder outlet obstruction (BOO) (panels D, H, L, P). The expression of ecto-NTPDase1 and 2 enzymes decreases in the detrusor of BOO patients compared to control individuals. Ecto-NTPDase3 was absent from detrusor samples of both groups of individuals, controls and BOO patients. No changes in ecto-5'nucleotidase/CD73 immunoreactivity seem to occur between the control group and patients with BOO.

Figure 14B shows the time course of the extracellular catabolism of ADP in urothelium-denuded detrusor strips from control organ donors and BOO patients. Detrusor strips from control subjects hydrolyze extracellular ADP (30 µM) with a half-degradation time ($t_{1/2}$) of 19±3 min (n=4), but this time increases (P<0.05) to 34±9 min (n=3) in patients with BOO due to BPH. Deficient extracellular ADP catabolism is compatible with the reduced amounts of ecto-NTPDases 1 and 2 in the detrusor of BPH patients compared to control individuals. The ADP metabolites detected in the bath were AMP, adenosine, inosine and hypoxanthine, whose concentrations increased with time. The concentration of ADP metabolites rose faster in control samples than in the detrusor from BOO patients. Interestingly, AMP concentrations in

the bathing fluid were higher than adenosine during the first 15 min following ADP (30 μM) applications (Figure 14B). This pattern indicates that (1) ecto-5'nucleotidase/CD73 (EC 3.1.3.5) is the rate limiting step of adenosine formation from the extracellular catabolism of adenine nucleotides in the human detrusor and that (2) this enzyme may be feed-forwardly inhibited by high levels of ADP in the extracellular milieu, as verified in many other tissues (*e.g.* neuromuscular junction, myenteric plexus, hippocampus) (Magalhães-Cardoso et al., 2003; Vieira et al., 2014; Cunha et al., 1992).

Role of the ecto-NTPDase2 localized on cholinergic nerves of the bladder of BOO patients

Inhibition of ecto-NTPDases with ARL67156 (100 μM) and POM-1 (100 μM) decreased [^{3}H]ACh from stimulated cholinergic nerve terminals innervating the human detrusor (Figure 15A). ARL67156 (100 μM) is a preferential inhibitor of ecto-NTPdase1, without any effect on ecto-NTPDase2 (Lévesque et al., 2007). To prevent ecto-NTPdase2 activity we used POM-1, a sodium polyoxotungstate exhibiting high affinity for ecto-NTPDases 1, 2 and 3 in recombinant cells (Wall et al., 2008). While both drugs exhibited a similar potency in bladder samples from control individuals, *i.e.* when ARL67156 (100 μM) and POM-1 (100 μM) were applied 15 min before S_2 (second stimulus) the amount of evoked [^{3}H]ACh release decreased by 34±5% (n=5) and 29±4% (n=4), respectively, the magnitude of the inhibitory effect of ARL67156 (100 μM) decreased to 13±8% (n=5) in the detrusor of BOO patients but the same did not occur with POM-1 (100 μM, 24±6%, n=5).

Taken together, these results suggest that inhibition of ecto-NTPDase1 (ATP diphosphohydrolase or apyrase) becomes functionally irrelevant accompanying the drop in NTPDase1 immunoreactivity in the detrusor of BOO patients (see Figure 14A). Despite we also observed a decline in ecto-NTPDase2 immunoreactivity in detrusor smooth muscle fibers of BOO patients, inhibition of this enzyme may still control evoked [^{3}H]ACh release because a subset of ecto-NTPDase2 co-localizes with the vesicular ACh transporter (VAChT) in cholinergic nerve fibers innervating the detrusor (Figure 15B), where it can mediate ADP accumulation from the hydrolysis of released ATP.

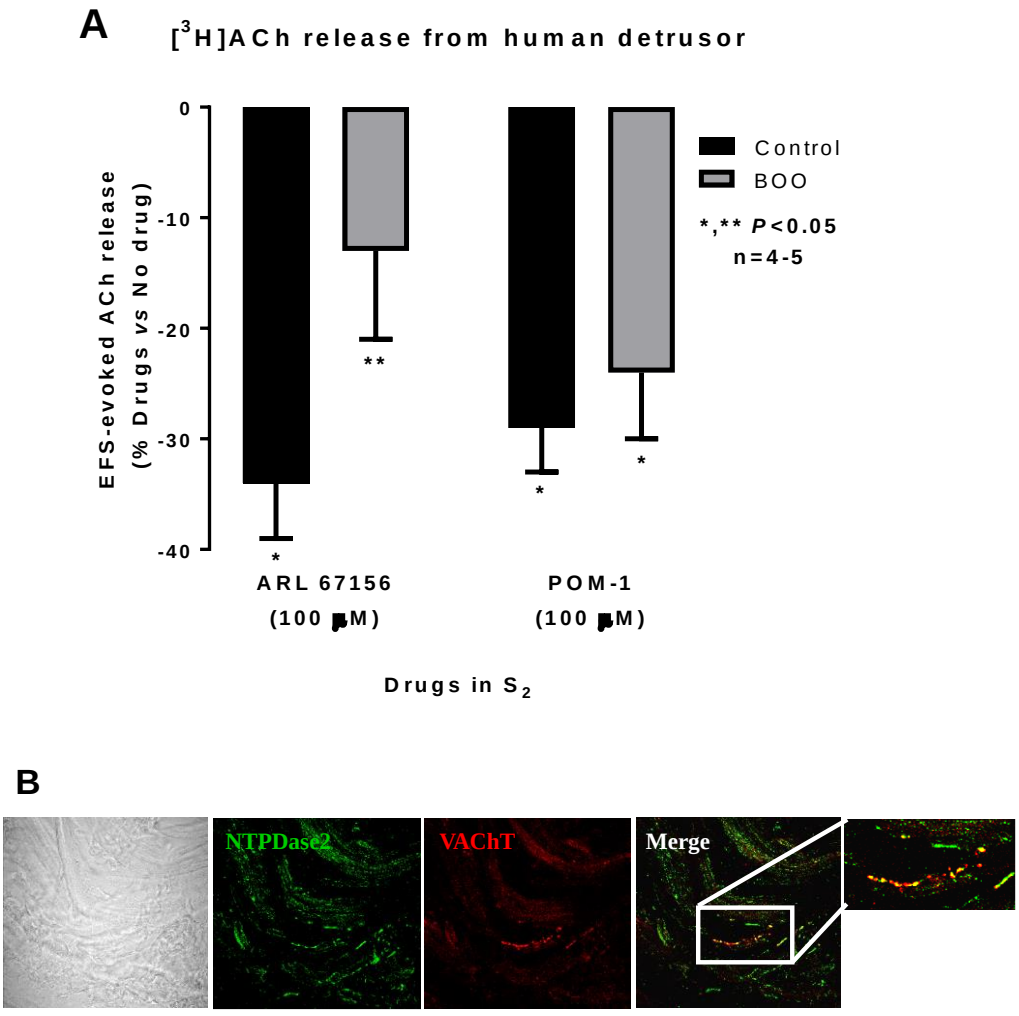


Figure 15. (A) Inhibition of NTPDases activity with ARL67156 and POM-1 decreases electrically evoked [^{3}H]ACh release from detrusor strips of cadaveric organ donors (control) and BOO patients. ARL67159 (100 µM) and POM-1 (100 µM) were applied 15 minutes before S_2. The ordinates are changes in S_2/S_1 ratios compared to the S_2/S_1 ratio obtained without addition of any drug. Data are means ± SD of four to five individuals. *P<0.05 (unpaired Student's t test with Welch's correction) represents significant differences when compared with zero percent of change or with the effect of the same drug in control individuals, respectively. **(B)** Co-localization of NTPDase2 with vesicular acetylcholine transporter (VAChT) in the human detrusor from control subject; to facilitate visualization of small cholinergic nerve terminals staining for VAChT (red) images correspond to the intensity projections over Z axis of five to six confocal microscopy stacks taken at the smooth muscle layer. VAChT-positive cholinergic nerve terminals (red) stained positively with antibody against the NTPDase2 (green); yellow staining denotes co-localization. Scale bars = 100 µm.

ADP accumulation in the detrusor of men with BOO due BPH contributes to attenuate cholinergic hyperactivity via $P2Y_{12}$ receptors activation

To investigate the activity of ADP-sensitive receptors on [^{3}H]ACh release from stimulated human detrusor samples, we used the enzymatically-stable ADP analogue, ADPβS (100 μM), which activates $P2Y_1$ (Shachter and Harden, 1997; Waldo et al., 2002), $P2Y_{12}$ (Herbert and Savi, 2003) and $P2Y_{13}$ receptors (Marteau et al., 2003). ADPβS (100 μM, applied 15 min before S_2) was devoid of effect on evoked [^{3}H]ACh release from the detrusor of control individuals, but it significantly (P<0.05) decreased transmitter release by 23±4% (n=7) in detrusor samples from men with BOO due to BPH (Figure 16A). The inhibitory effect of ADPβS (100 μM) was fully reversed by selectively blocking the $P2Y_{12}$ receptor with AR-C66096 (100 nM), when the antagonist was present throughout the experimental period (*i.e.* both in S_1 and S_2).

To evaluate the endogenous role of ADP-sensitive receptors on [^{3}H]ACh release from detrusor strips isolated from BPH patients, we used MRS2179 (300 nM), AR-C66096 (100 nM) and MRS2211 (10 μM) which selectively antagonize $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptors, respectively. Blockade of $P2Y_1$ and $P2Y_{13}$ receptors with MRS2179 (300 nM) and MRS2211 (10 μM) did not significantly change the evoked transmitter release from detrusor samples of obstructed patients (Figure 16B). This contrasts with the signiticant (P<0.05) facilitatory effect observed upon blocking the $P2Y_{12}$ receptor with the AR-C66096 (100 nM).

Overall, these results indicate that endogenous ADP accumulation at the neuromuscular synapse of the obstructed human detrusor contributes to attenuate cholinergic hyperactivity through the activation of $P2Y_{12}$ receptors.

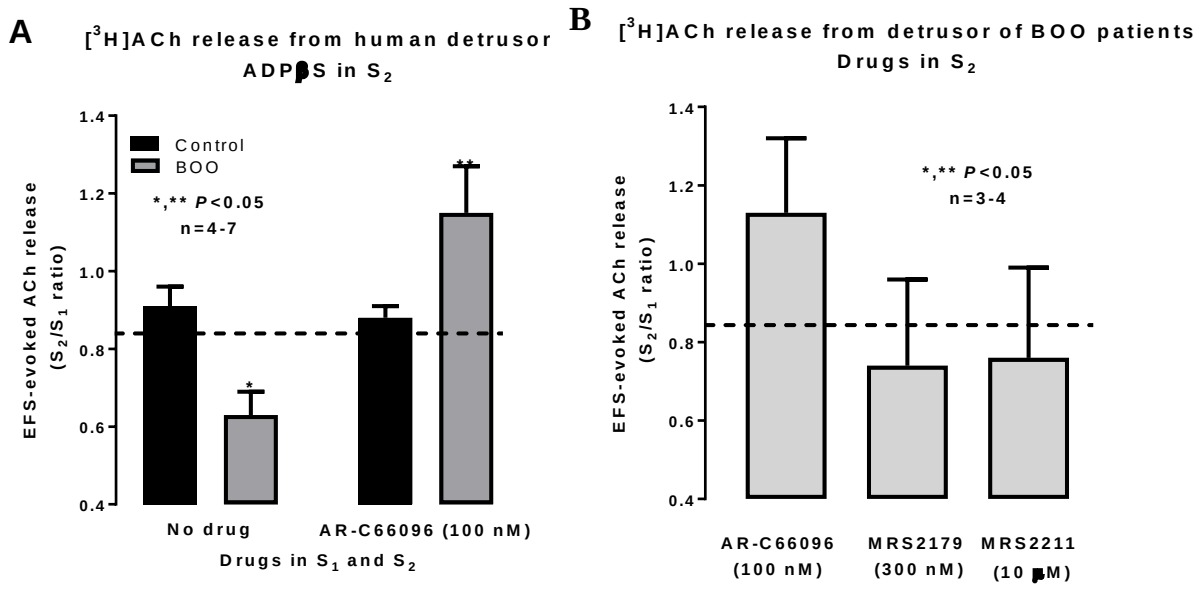


Figure 16. (A) Activation of ADP-sensitive receptors with ADPβS, an enzymatically-stable ADP analogue, inhibits [^{3}H]ACh release from electrical stimulated detrusor strips of control individuals and patients with BOO due to BPH. ADPβS (100 μM) was added 15 minutes before S_2 and the $P2Y_{12}$ receptor antagonist, AR-C66096 (100 nM), was present throughout assay, including S_1 and S_2. Ordinates indicate changes in S_2/S_1 in presence of ADPβS (100 μM) compared to corresponding S_2/S_1 in control conditions (dashed horizontal line). In the absence of added drugs the S_2/S_1 ratio was 0.83 ± 0.05 in controls and 0.84 ± 0.04 in patients with BOO. Data are means ± SD of four to seven individuals. *,** *P*<0.05 (unpaired Student's t test with Welch's correction) represents significant differences when compared with zero percent of change or with the effect of the same drug in control individuals, respectively. **(B)** Effects of the blockade of $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptors with MRS2179 (300 nM), AR-C66096 (100 nM) and MRS2211 (10 μM) on evoked [^{3}H]ACh release from detrusor samples from patients with BOO due to BPH. AR-C66096, MRS2179 and MRS2211 were added to the incubation media 15 minutes before S_2. The ordinates are changes in S_2/S_1 ratio compared to the S_2/S_1 ratio obtained without addition of any drug. Data are means ± SD of three to four individuals.

Localization of ADP-sensitive receptors in the human urinary bladder from control subjects and patients with bladder outlet obstruction

Figure 17A shows confocal microscopy images of human detrusor samples isolated from control individuals (panels B, F, J) and from patients with BOO due BPH (panels D, H, L) stained against ADP-sensitive receptors, $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$. The smooth muscle fibers of the human detrusor are endowed with $P2Y_1$ and $P2Y_{12}$ receptors, but we did not find $P2Y_{13}$ receptor immunoreactivity. While no

significant changes were detected concerning the $P2Y_1$ immunoreactivity between control individuals (Figure 17A(B)) and patients with BOO due to BPH (Figure 17A(D)), the $P2Y_{12}$ immunofluorescence staininig increased substantially in samples from obstructed patients (compare Figures 17A(F) and 17A(H)).

In addition to the presence of $P2Y_{12}$ immunoreactivity in detrusor smooth muscle fibers (green staining), Figure 17B shows that this receptor is also present on VAChT-positive cholinergic nerve fibers (red stainig) denoted by the yellow staining resulting from merging the two channels in the confocal microscope. This finding supports the predominant neuromodulatory role of the $P2Y_{12}$ compared to the other ADP-sensitive receptors in obstructed human detrusor samples.

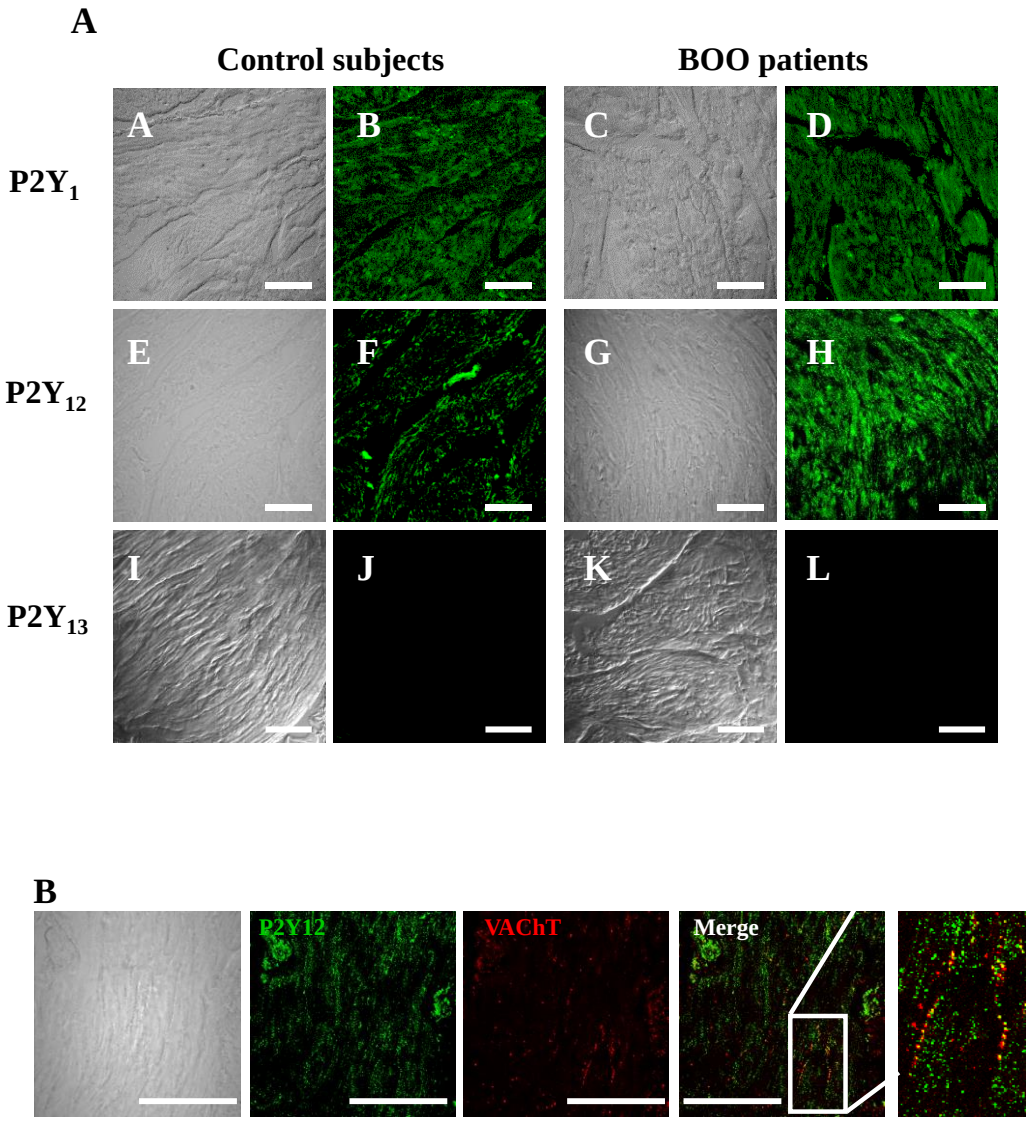


Figure 17. (A) Tissue distribution of ADP-sensitive receptors (green staining), $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptors, in transverse sections of the detrusor of control organ donors (panels B, F, J) and of patients with bladder outlet obstruction (BOO) due to benign prostate hyperplasia (BPH) (D, H, L). Black and white images are differential interference contrast (DIC) images. Data represent four individuals per group, including organ donors and men with BOO. Scale bars: 100 µm. **(B)** Co-localization experiments of the $P2Y_{12}$ receptor and vesicular acetylcholine transporter (VAChT) immunoreactivity in human detrusor samples from control subjects bladder. A subset of $P2Y_{12}$ receptors (green staining) co-localizes with VAChT (red staining) on cholinergic nerve fibers. To facilitate visualization of small nerve fibers stained with VAChT images correspond to higher magnification intensity projections over Z axis of six confocal microscopy stacks. Scale bars: 50 µm.

ATP acting via P2X2/3 heterodimers favours cholinergic hyperactivity in obstructed human detrusor strips

As a consequence of deficits in the expression and activity of ecto-NTPDase 1 and 2 ATP may accumulate in the detrusor of patients with BOO due to BPH compared to control individuals (Silva-Ramos et al., 2015a; see also Figure 14A this chapter). Using myographic recordings, it was demonstrated that ATP accumulated in the bladder contributes to increase detrusor responsiveness via the activation of overexpressed P2X1 receptors on smooth muscle fibers (Silva-Ramos et al., 2016b), but no studies were performed so far to investigate the role of ionotropic P2X receptors in the control of cholinergic neurotransmission and whether they interfere with the inhibitory control mediated by ADP (via $P2Y_{12}$ receptor activation) on evoked [^{3}H]ACh release from obstructed detrusor samples.

The non-selective P2 receptors antagonist, PPADS (10 µM), decreased by 43±6% (n=4, P<0.05) the evoked release of [^{3}H]ACh in the detrusor of men with BOO due BPH, without affecting the transmitter release (6±8%, n=4) in control individuals (Figure 18). A similar trend was observed by blocking selectively P2X2/3 receptor heterodimers with TNP-ATP (10 nM). This drug was more effective in decreasing evoked [^{3}H]ACh release in the detrusor of obstructed patients (43±16%, n=4) than in control organ donors (Figure 18). The selective P2X3 antagonist, A317491 (100 nM), attenuated the release of [^{3}H]ACh from the detrusor by 20±2% (n=4) and 24±4% (n=5) in control individuals and in BOO patients, respectively (Figure 18). Results indicate that surplus ATP acting via P2X2/3 heterodimers favours cholinergic hyperactivity in obstructed human bladders.

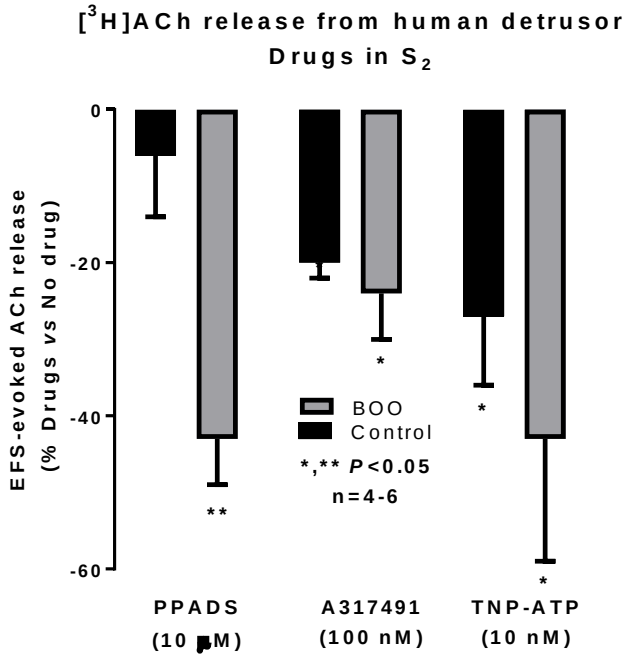


Figure 18. Effects of PPADS (10 µM), A317491 (100 nM) and TNP-ATP (10 nM) on electrically evoked [^{3}H]ACh release from urothelium-denuded detrusor strips from cadaveric organ donors (control) and men with bladder outflow obstruction (BOO) due benign prostatic hyperplasia (BPH). Drugs were applied 15 minutes before S_2. The ordinates are changes in S_2/S_1 ratio compared to the S_2/S_1 ratio obtained without addition of any drug. **,**P*<0.05 (unpaired Student's t test with Welch's correction) represents significant differences when compared with zero percent of change or with the effect of the same drug in control individuals, respectively.

Tonic activation of ADP-sensitive inhibitory P2Y$_{12}$ receptors restrains P2X2/3-mediated excitation of cholinergic nerves in obstructed detrusor samples

Taking into consideration that the inhibitory $P2Y_{12}$ receptor-mediated tonus increases in the detrusor of men with BOO due to BPH along with cholinergic hyperexcitation operated by surplus ATP accumulation via P2X2/3 heterodimers, we thought it was important to investigate the interplay between these two purinoceptors in the control of [^{3}H]ACh release in the obstructed human bladder. Figure 19 shows that selective blockade of the $P2Y_{12}$ receptor with AR-C66096 (100 nM) fully prevented (7±13%, n=4) the inhibitory effect of the P2X2/3 antagonist, TNP-ATP (10 nM, -43±16 %, n=4) on evoked [^{3}H]ACh release from detrusor strips of BOO patients.

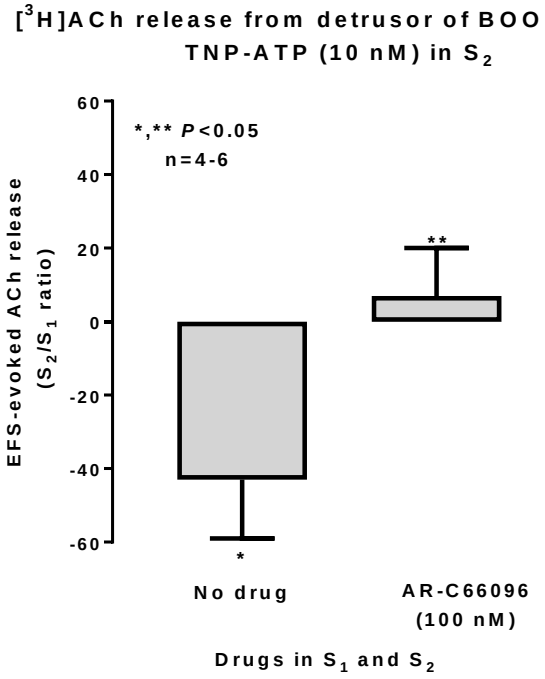


Figure 19. Blockade of the $P2Y_{12}$ receptor with AR-C66096 (100 nM) prevented the inhibitory effect of TNP-ATP (10 nM, a P2X2/3 receptor antagonist) on [^{3}H]ACh release from stimulated detrusor strips of BOO patients. TNP-ATP (10 nM) was added 15 min before S_2 and AR-C66096 (100 nM) was present throughout assay, including S_1 and S_2. The ordinates are changes in S_2/S_1 ratio compared to the S_2/S_1 ratio obtained without addition of any drug. Data are mean ± SD of four to six individuals. *,**P<0.05 (unpaired Student's t test with Welch's correction) represents significant differences when compared to the situation where no drugs were added to the incubation solution or to the effect of TNP-ATP in the absence of AR-C66096.

DISCUSSION

Mounting evidence indicate that deregulation of the purinergic signaling pathway is involved in urinary bladder disorders (review by Burnstock, 2014; Rapp et al., 2005), both in humans (Palea et al., 1993; Silva-Ramos et al., 2015a; Silva et al., 2015) and in experimental animal models (Timóteo et al., 2014; Banks et al., 2006; Birder et al., 2006; Calvert et al., 2001). There is however gaps in our knowledge concerning the key purinergic players underlying urinary bladder disorders and how the information made available might contribute to the rehabilitation of the normal bladder function using drugs targeting the purinergic signaling pathway.

In a previous study, our group showed that impairment of ecto-NTPDases activity in detrusor strips from patients with BOO due BPH contributes to increase extracellular ATP content in the bladder leading to cholinergic hyperactivity, also because adenosine formation from released adenine nucleotides by ecto-5'nucleotidase/CD73 and, subsequent, activation of prejunctional inhibitory A_1 receptors is deficient (Silva-Ramos et al., 2015a). Here, we add some information indicating that along with the dominant extracellular ATP catabolism by ecto-NTPDase1/CD39 (also called ATP diphosphohydrolase or ecto-apyrase, EC 3.6.1.5) converting the nucleotide directly into AMP (Silva-Ramos et al., 2015a), ATP dephosphorylation into ADP by ecto-NTPDase2 (CD39L1, ATPase, EC 3.6.1.3) may also play a role when ecto-NTPDase1/CD39 is deficient or inhibited, as verified in the obstructed human detrusor. Localization of ecto-NTPDase2 on VAChT-positive cholinergic nerve terminals in the human detrusor indicates that ADP formation from released / accumulated ATP (Silva et al., 2015) may reach high enough levels to modulate cholinergic neurotransmission in obstructed human bladders. As a matter of fact, our results show here for the first time that endogenous ADP (and its enzymatically-stable analogue, ADPβS) downregulates [^{3}H]ACh release from stimulated cholinergic nerves through the activation of prejunctional inhibitory $P2Y_{12}$ receptors in obstructed human bladders. Interestingly, these receptors co-localize with the $P2Y_1$ receptor (another ADP-sensitive receptor) on smooth muscle fibers of the human detrusor, but only the $P2Y_{12}$ receptor is upregulated in the detrusor of men with BOO due BPH.

The kinetics of the extracellular ATP and ADP catabolism is 1.4 and 1.9 times slower in the detrusor of BOO patients than controls, respectively (Silva-Ramos et al., 2015a; this study). This difference is mainly attributed to decreases in ecto-NTPDase1/CD39 expression and activity converting ATP and ADP directly into AMP, with the subsequent dephosphorylation of AMP to adenosine by ecto-5'nucleotidase/CD73 in smooth muscle fibres. Minor changes were detected in the expression of ecto-NTPDase2 bound to VAChT-positive cholinergic nerve terminals, which might favour transient ADP accumulation at the detrusor cholinergic neuromuscular synapse of patients with BOO due BPH. This was hypothesized because ecto-NTPDase2 is a nucleoside triphosphatase hydrolyzing ATP 10-15 times more efficiently than ADP (Matsuoka and Ohkubo, 2004). Conversely,

NTPDase3 seems to be absent from the human detrusor as demonstrated by the lack of immunoreactivity detected by confocal microscopy using a highly specific NTPDase3 antibody. A similar distribution pattern of ecto-NTPDases was found in the mouse bladder, where immunolocalization studies showed that ecto-5nucleotidase/CD73 is present in detrusor smooth muscle fibers together with the ecto-NTPDase1/CD39 (Yu et al., 2011). The presence of ecto-NTPDase2 on VAChT-positive cholinergic nerve terminals was also observed in the rat detrusor (Carneiro et al., 2014).

The functional role of NTPDases 1 and 2 on evoked [^{3}H]ACh release from the human bladder was evaluated using subtype-specific inhibitors. ARL67159 (100 μM) is a weak competitive inhibitor of NTPDase1, NTPDase3 and NPP1, without any effect on NTPDase2 and ecto-5'nucleotidase (Lévesque et al., 2007). ARL67159-induced inhibition of [^{3}H]ACh release from the human detrusor was much more evident in control individuals than in patients with BOO due to BPH. On the other hand, POM-1 (100 μM), a potent inhibitor of all NTPDases including NTPDase2 (Wall et al., 2008), had a similar inhibitory effect on transmitter release from both groups of individuals. The lack of the inhibitory effect of ARL67156 on evoked [^{3}H]ACh release from obstructed human detrusor samples suggests that it might depend on transient ADP accumulation and activation of ADP-sensitive P2Y receptors on cholinergic nerve terminals.

ADP is the endogenous agonist of $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptors, as it binds to these receptors with greater affinity than ATP (Palmer et al., 1998; Boeynaems et al., 2003; Marteau et al., 2003). At the $P2Y_1$ receptor, ADP and its analogues tend to be full agonists (the EC_{50} of ADP is ~100 nM), whereas ATP may act as a partial agonist (Abbracchio et al., 2006). At the $P2Y_{12}$ receptor, ADP and its analogues activate (the EC_{50} of ADP is ~100 nM) and 5'-triphosphate derivatives antagonize the receptor (Gachet and Hechler, 2005). At the $P2Y_{13}$ receptor, both ADP and ATP might act as full agonists, with EC_{50} values of ~100 nM (Abbracchio et al., 2006). ADPβS is an enzymatically-stable ADP analogue and a potent agonist of $P2Y_1$ (EC_{50} = 96 nM), $P2Y_{12}$ (EC_{50} = 82 nM) (Jacobson et al., 2002) and $P2Y_{13}$ (EC_{50} = 42 nM) (Communi et al., 2001) receptor subtypes. In this study, we used ADPβS at a higher concentration (100 μM) in order to activate all ADP-sensitive receptors. ADPβS decreased [^{3}H]ACh release from stimulated detrusor strips isolated from

BOO patients, but it was without effect in preparations from control individuals. The inhibitory effect of ADPβS was fully reverserd upon blocking the $P2Y_{12}$ receptor with AR-C66096. On its own, blockage of the $P2Y_{12}$ receptor facilitated [^{3}H]ACh outflow from obstructed human detrusor samples, but the same did not occur when $P2Y_1$ (MRS2179) and $P2Y_{13}$ (MRS2211) receptor antagonists were used instead of AR-C66096. The presence of $P2Y_{12}$ immunoreactivity on VAChT-positive cholinergic nerve fibers and the overexpression of this receptor in detrusor samples from BPH patients strengthen our theory that transient endogenous ADP accumulation in the bladder of BPH patients may partially control cholinergic hyperactivity through tonic activation of inhibitory $P2Y_{12}$ receptors.

The role of $P2Y_1$ and $P2Y_{12}$ receptors localized on smooth muscle fibers of the human detrusor remains to be determined. Activation of $P2Y_{12}$ receptors in smooth muscle fibers induces bladder contractions in mice (Yu et al., 2014). The presence of $P2Y_1$ receptors was also demonstrated by RT-PCR analysis in smooth muscle cells of the rat detrusor (Obara et al., 1998). Our group showed that $P2Y_1$ receptors are localized in VAChT-positive cholinergic nerves of the rat urinary bladder, where they may counteract bladder overacitivty caused by activation of UDP-sensitive $P2Y_6$ receptors (Carneiro et al., 2014). We found no significant immunoreactivity against the $P2Y_{13}$ receptor in the detrusor of both control individuals and BPH patients. The absence of $P2Y_{13}$ receptors in the human bladder was confirmed by RT-PCR analysis (Communi et al., 2001). These findings indicate that the $P2Y_{13}$ receptor is not involved in the control of cholinergic neurotransmission in the human detrusor, notwithstanding the fact that it has been involved in the presynaptic control of ACh release at the mouse motor endplate (Guarracino et al., 2016).

In 1972, Burnstock showed for the first time that ATP contributes along with ACh to the contractile responses produced by parasympathetic nerve stimulation in the urinary bladder (Burnstock, 1972). Later on, it was demonstrated that ionotropic P2X receptors, namely P2X3 or/and P2X2/3 subtypes, mediate sensory functions in the lower urinary tract (Rapp et al., 2005; Ford et al., 2006). The voiding reflex involves ATP release from urothelial cells during bladder distension, which initiates the micturition reflex by activating P2X3 receptors on suburothelial sensory nerve afferents (Calvert et al., 2008). On the other hand, ATP also activates P2X1 receptors located in the detrusor smooth muscle to initiate bladder contractions

during voiding (Burnstock, 1999; Cockayne et al., 2000). Accumulation of extracellular ATP has been reported in lower urinary tract disorders (Smith et al., 2005), such as overactivity (Silva-Ramos et al., 2013) and obstructed (Silva et al., 2015; Silva-Ramos et al., 2016a) bladders. Overexpression of the P2X3 receptor has also been documented in bladder disorders (Brady et al., 2004), including some types of neurogenic bladder overactivity. These changes are suggested to be responsible for unstable bladder activity (Rapp et al., 2005; Ruggieri, 2006). Due to the role of these receptors in the pathophysiology of overactive bladder syndromes, several drugs have been tested in models of rat bladder disorders. For instance, the selective P2X3 receptor antagonist, A317491, has been proposed for the treatment of interstitial cystitis (Ito et al., 2008).

In this study, we also decided to evaluate the effect of subtype-specifc P2X receptor antagonists on evoked [^{3}H]ACh release from the detrusor of control individuals and from patients with BOO due to BPH. To this end, we used pyridoxaphosphate-6-azophenyl-29.59-disulfonic acid (PPADS, 10 μM, a non-selective P2 antagonist receptor), A317491 (100 nM, a P2X3 receptor antagonist), and TNP-ATP (10 nM, a preferential P2X2/3 receptor antagonist); the latter compound has nanomolar affinity for fast desensitizing P2X1, P2X2/3 and P2X3 receptors (Virginio et al., 1998; Jacobson et al., 2002; Jarvis et al., 2010). Both, PPADS and TNP-ATP, inhibited (by about 40%) [^{3}H]ACh release from detrusor strips of men with BOO due to BPH, but the two drugs had smaller inhibitory effects in control preparations. The selective P2X3 receptor antagonists, A317491 (Jarvis et al., 2002), produced the smallest inhibitory effect on evoked [^{3}H]ACh release of the three tested antagonists. Taken together these results suggest that endogenous ATP facilitates cholinergic hyperactivity in obstructed human detrusor samples through the activation of TNP-ATP-sensitive excitatory P2X2/3 receptors.

Numerous studies demonstrate (1) the existence of functional interactions between co-localized ionotropic P2X and metabotropic P2Y receptors, and that (2) the crosstalk between P2 purinoceptor subtypes may be affected under pathological conditions (James and Butt, 2002; Birder et al., 2004; Tempest et al., 2004). A recent study from our group showed that activation of metabotropic $P2Y_6$ and ionotropic P2X3 receptors cooperate to increase nonneuronal ATP and [^{3}H]ACh release from urothelial strips with the *lamina propria* attached isolated from control organ donors

(Silva et al., 2015). In the urinary bladder ionotropic P2X3 receptors can function as homomultimeric P2X3 and heteromultimeric P2X2/3 channels (Cockayne et al., 2005). Our data show here for the first time that [^{3}H]ACh release from stimulated detrusor samples of patients with BOO due to BPH is modulated both positively and negatively by P2X2/3 heterodimers and by ADP-sensitive inhibitory $P2Y_{12}$ receptors, respectively. While the ATP-mediated excitation of cholinergic nerve fibers via P2X2/3 receptors is already verified in control individuals, the superfluous [^{3}H]ACh release observed in obstructed detrusor samples may be (at least partially) downmodulated by the enzymatic breakdown of ATP into ADP by NTPDase2 and, subsequent, activation of prejunctional inhibitory $P2Y_{12}$ receptors (see above). Although this sequence of events seems to be highly probable, we decided to test the functional repercussions of the interplay between these two purinoceptor subtypes on cholinergic hyperactivity observed in the detrusor of BPH patients. Data show that the inhibitory role of the P2X2/3 antagonist, TNP-ATP, on evoked [^{3}H]ACh release was fully prevented by bloking activation of the $P2Y_{12}$ receptor with AR-C66096. Thus, results strongly indicate that tonic activation of ADP-sensitive inhibitory $P2Y_{12}$ receptors restrains the P2X2/3-mediated excitation of cholinergic nerves in obstructed detrusor samples.

CONCLUSION

In conclusion, data suggest that drugs that favour ADP accumulation in the cholinergic neuromuscular synapse of the human detrusor and/or that promote activation of overexpressed inhibitory $P2Y_{12}$ receptors on cholinergic nerve fibers may be useful to counteract OAB symptoms associated with BOO due to BPH. Likewise, blockade of fast desensitizing homomultimeric P2X3 and heteromultimeric P2X2/3 ionotropic receptors may also be used to cease bladder overactivity by acting on both sides (sensory and motor, respectively) of the micturition reflex.

ACKNOWLEDGEMENTS

This research was partially supported by Fundação para a Ciência e a Tecnologia (FCT, FEDER funding, projects PTDC/SAU-OMS/104369/2008, REEQ/1168/SAU/2005, REEQ/1264/SAU/2005, PEst-OE/SAU/UI0215/2014 and UID/BIM/4308/2016), Associação Portuguesa de Urologia (APU), and University of Porto/Caixa Geral de Depósitos (Investigação Científica na Pré-Graduação). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. IS was in receipt of a PhD Studentship from FCT (SFRH/BD/88855/2012). The authors wish to thank Mrs. Helena Costa e Silva and Belmira Silva for their valuable technical assistance.

PAPER 3

American Journal of Physiology – Renal Physiology, 2017, 313(2): F388- F403.
DOI: 10.1152/ajprenal.00392.2016

Inhibition of cholinergic neurotransmission by β_3-adrenoceptors depends on adenosine release and A_1 receptors activation in human and rat urinary bladders

Isabel Silva[1,2], Ana Filipa Costa[1,2], Sílvia Moreira[1,2], Fátima Ferreirinha[1,2], Maria Teresa Magalhães-Cardoso[1,2], Isabel Calejo[1,2], Miguel Silva-Ramos[1,2,3] and Paulo Correia-de-Sá[1,2]*

[1]Laboratório de Farmacologia e Neurobiologia, [2]Center for Drug Discovery and Innovative Medicines, Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto and [3]Serviço de Urologia, Centro Hospitalar do Porto, Porto, Portugal

* Corresponding author: farmacol@icbas.up.pt

Contribution of authors: PCS supervised the project, designed the experiments, and analyzed data. IS, MSR and PCS wrote the paper. IS, AFC, IC and MTMC performed and analyzed data from enzymatic kinetic experiments by HPLC. IS, AFC and MSR performed and analyzed data from acetylcholine and adenosine release experiments. AFC, SM and IS performed and analysed data from in vivo rat bladder cystometry experiments. IS and FF performed Western blot analysis, immunofluorescence staining and confocal microscopy observations. MSR recruited the patients and collected bladder samples. IS, AFC, SM, MTMC, FF, MSR and PCS interpreted data, discussed the clinical implications and commented on the manuscript at all stages.

ABSTRACT

The direct detrusor relaxant effect of β_3-adrenoceptor agonists as a primary mechanism to improve overactive bladder symptoms has been questioned. Among other targets, activation of β_3-adrenoceptors down-modulate nerve-evoked acetylcholine (ACh) release, but there is insufficient evidence for the presence of these receptors on bladder cholinergic nerve terminals. Our hypothesis is that adenosine formed from the catabolism of cyclic AMP in the detrusor may act as a retrograde messenger via prejunctional A_1 receptors to explain inhibition of cholinergic activity by β_3-adrenoceptors. Isoprenaline (1 μM) decreased [^{3}H]ACh release from stimulated (10 Hz, 200 pulses) human (-47±5%) and rat (-38±1%) detrusor strips. Mirabegron (0.1 μM, -53±8%) and C316,243 (1 μM, -37±7%) mimicked isoprenaline (1 μM) inhibition and their effects were prevented by blocking β_3-adrenoceptors with L748,337 (30 nM) and SR59230A (100 nM), respectively in

human and rat detrusor. Mirabegron and isoprenaline increased extracellular adenosine in the detrusor. Blockage of A_1 receptors with 1,3-dipropyl-8-cyclopentylxantine (DPCPX, 100 nM) or the equilibrative nucleoside transporters (ENT) with dipyridamole (0.5 μM) prevented mirabegron and isoprenaline inhibitory effects. Dipyridamole prevented isoprenaline-induced adenosine outflow from the rat detrusor and this effect was mimicked by the ENT1 inhibitor, S-(4-nitrobenzyl)-6-thioinosine (NBTI, 30 μM). Cystometry recordings in anaesthetized rats demonstrated that SR59230A, DPCPX, dipyridamole and NBTI reversed the decrease in the voiding frequency caused by isoprenaline (0.1 – 1000 nM). Data suggest that inhibition of cholinergic neurotransmission by β_3-adrenoceptors results from adenosine release via equilibrative nucleoside transporters and prejunctional A_1 receptors stimulation in human and rat urinary bladder.

Keywords: Human urinary bladder; Adenosine release; Acetylcholine release; Adenosine A_1 receptors; β_3-adrenoceptor.

INTRODUCTION

The clinical success of β_3-adrenoceptor agonists, like mirabegron, for the management of overactive bladder (OAB) syndrome has generated a great interest in their mechanisms of action (Andersson, 2016; Thiagamoorthy et al., 2015). Stimulation of β_3-adrenoceptors reduce basal bladder tension (decreasing spontaneous microcontractions in vitro and non-voiding contractions in vivo) and relax bladder strips precontracted with carbachol or KCl (Igawa et al., 1999; Takeda et al., 1999; Wuest et al., 2009). Whether the direct relaxant effect of β_3-adrenoceptor agonists on detrusor smooth muscle is the primary underlying mechanism to improve OAB symptoms has increasingly been questioned (Gillespie et al., 2015; Igawa and Michel, 2013; Persyn et al., 2016). The key argument is that the peak plasma levels of mirabegron when administered in therapeutic (50 mg daily) doses (83 – 167 nM) are below its potency (EC50 ~1-3 μM) to relax detrusor smooth muscle strips (reviewed in Michel and Korstanje, 2016). Supporting the view that mechanisms other than direct effects on detrusor muscle might contribute, mirabegron dose-dependently decreases the activity of Aδ- and C-fibers of primary nerve afferents in

response to bladder filling (Aizawa et al., 2015) and activation of β_3-adrenoceptors inhibits neurogenic contractions of rat and human urinary bladder samples (Rouget et al., 2014).

Recent evidence suggest that activation of β_3-adrenoceptors down-modulates nerve-evoked acetylcholine (ACh) release in the human bladder (D'Agostino et al., 2015). The existence of β_3-adrenoceptors in the human detrusor has been postulated in 1998 and their expression at the mRNA levels was confirmed later on by RT-PCR (Igawa et al., 1998; Igawa et al., 1999). Nomiya and Yamaguchi (2003) claimed that the β_3-adrenoceptor accounts for more than 95% of all β-adrenoceptor mRNA in the human bladder (Nomiya and Yamaguchi, 2003; reviewed in Michel and Vryday, 2006), but such difference has been questioned in a more recent quantitative genomic resource describing proteomes and transcriptomes across diverse tissues and organs of the human body (Uhlén et al., 2015). Immunolocalization studies showed that β_3-adrenoceptors were present in the urothelium, interstitial cells and smooth muscle fibers of human (Limberg et al., 2010; Otsuka et al., 2013) and rat (Kullmann et al., 2009) bladders, but there is contradictory evidence for these receptors being expressed on cholinergic nerve terminals (Coelho et al., 2017; Otsuka et al., 2008). Therefore, the mechanism by which β_3-adrenoceptors operate inhibition of evoked ACh release from cholinergic nerve terminals in the urinary bladder remains unknown.

On the molecular level, β_3-adrenoceptors couple mainly to stimulatory G proteins (Gs), thereby activating adenylyl cyclase to generate intracellular cyclic AMP. Besides its multiple downstream intracellular effects, which may involve modulation of potassium channels to cause hyperpolarization of the plasma membrane (Igawa and Michel, 2013), cyclic AMP may be degraded into AMP and adenosine intracellularly or can be actively pumped out from cells contributing to extracellular adenosine formation, in a process that depends on the sequential activity of ecto-nucleotide pyrophosphatase/phosphodiesterase 1 (ecto-PDE, alias ENPP1) and ecto-5'-nucleotidase/CD73 (see *e.g. ref.* Chiavegatti et al., 2008; Sassi et al., 2014). This raises the intriguing question of whether the urinary bladder, like many other tissues, provides its own source of extracellular adenosine originated from the catabolism of intracellular cyclic AMP and if such a mechanism is responsible for the β_3-adrenoceptor inhibitory control of cholinergic neurotransmission in the human detrusor.

Adenosine directly relaxes pre-contracted urinary bladder detrusor strips in different species (Acevedo et al., 1992; Brown et al., 1979; Burnstock et al., 1978; King et al., 1997; Nicholls et al., 1992), including humans (Rubinstein et al., 1998). Notwithstanding this, we recently showed that adenosine-induced relaxation of detrusor contractions requires high (millimolar) concentrations of the nucleoside compared to those require to decrease nerve-evoked ACh release from human detrusor strips (Silva-Ramos et al., 2015). Involvement of the A_1 receptor in the inhibitory action of adenosine on transmitter release from the stimulated human detrusor was confirmed both pharmacologically, using the selective A_1 receptor antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), and by confocal microscopy showing that the A_1 receptor co-localizes with VAChT-immunoreactivity on cholinergic nerve terminals of the humans urinary bladder. Taking this into account, we hypothesized that adenosine formed from the catabolism of cyclic AMP in the detrusor may act as a retrograde messenger via pre-junctional A_1 receptors to explain the inhibitory control of cholinergic activity by β_3-adrenoceptors in the humans and rat urinary bladder. We addressed this question by doing *in vitro* experiments using human and rat isolated urinary bladder strips to measure the release of [^{3}H]ACh and adenosine, to study the kinetics of the extracellular cyclic AMP catabolism by HPLC, and to evaluate the tissue distribution of β_3 and A_1 receptors in the urinary bladder by immunofluorescence confocal microscopy. In addition, we tested whether a similar phenomenon occurs to control the sensory bladder drive of the micturition cycle by β_3-adrenoceptors. To this end, we evaluated the urodynamic effects of selective β_3- and A_1-receptor antagonists on isoprenaline-induced inhibition of bladder activity caused by instillation of this drug into the bladder lumen of urethane-anaesthetized rats.

MATERIAL AND METHODS

Animals

Animal care and experimental procedures were in accordance with the guidelines prepared by Committee for the Update of the Guide for the Care and Use of Laboratory Animals (National Research Council, USA) and followed the European Communities Council Directive (86/609/EEC). All studies involving animals are

reported in accordance with the ARRIVE guidelines for reporting experiments involving animals. A total of 50 animals were used in the experiments described here. Male rats (Wistar, 200-300 g; Charler River, Barcelona, Spain) were kept at a constant temperature (21ºC) and a regular light (06:30-19:30 h) – dark (19:30-06:30 h) cycle, with food and water provided *al libitium*.

Human bladder samples

Samples of the human detrusor were collected from the bladder dome of ten male organ donors (46±4 years of age) at the time of harvesting organs for transplantation. Collected samples were immediately placed at 4-6ºC 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 $K_2HPO_4.3H_2O$, 15 mM KCl, and 10 mM $NaHCO_3$, pH 7.4) and transported to the laboratory. Experiments were performed within the first 24 h after collection, which corresponds to the tissue viability window. This study and all its procedures were approved by the Ethics Committees of CHP and ICBAS-UP and were authorized by the National Transplantation Committee. Regarding deceased organ donation, the legal frame work allows the “Presumed Consent” stating that residents in Portugal are consenting donors for transplantation and research unless the individual previously objected during his or her life. The investigation conforms to the principles outlined in *The Code of ethics of the World Medical Association* (Declaration of Helsinki).

Quantification of [^{3}H]ACh release

The experiments were performed in isolated detrusor muscle strips without the mucosa of both human and rat urinary bladders. The mucosa was dissected out either by blunt dissection through cleavage at the lamina propria or by gently rubbing the urothelium with a cotton wool swab in human and rat bladder samples, respectively (Carneiro et al., 2014; Silva-Ramos et al., 2015a). Full thickness isolated detrusor muscle strips (3 mm width, 5 mm length; weighting 6.32 ± 0.25 mg (human) and 5.91 ± 0.20 mg (rat)) were mounted in 365 µL capacity chambers of a Brandel SF-12 automated superfusion system (Valley Internationnal Corp., Austin, TX, USA) heated at 37ºC. Then, the preparations were continuously superfused with gassed (95% O_2 and 5% CO_2) Tyrode’s solution (pH 7.4) containing (mM): NaCl 137, KCL 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1, NaH_2PO_4 0.4, $NaHCO_3$ 11.9, glucose 11.2 and choline

0.001. After a 30 min equilibration period, cholinergic neurons were loaded during 40 min with 1 μM [^{3}H]choline (specific activity 5 μCi nmol^{-1}) under electrical field stimulation (1 Hz frequency, 0.2 ms pulse width, 75 mA) using two platinum-made grid electrodes placed above and below the muscle strip (transmural EFS stimulation). Following loading, the washout superfusion (1 ml/min) of the preparations was performed during 120 min with Tyrode's solution supplemented with the choline uptake inhibitor, hemicholinium-3 (10 μM). Tritium outflow was evaluated by liquid scintillation spectrometry (TriCarb2900TR, Perkin Elmer, and Boston, USA; % counting efficiency: 56 ± 2%) after appropriate background subtraction, using 1 ml bath samples automatically collected every 1 min using the SF-12 suprafusion system. [^{3}H]ACh release was evoked by two periods of EFS (S_1 and S_2), each consisting of 200 squares wave pulses of 0.2 ms duration delivered at 10-Hz frequency. Test drugs were added 6 min before S_2 and were present up to the end of the experiments. The evoked [^{3}H]ACh release was calculated by subtraction the basal tritium outflow from the total tritium outflow during the stimulation period (see *e.g.* Carneiro et al., 2014; Silva-Ramos et al., 2015a). In control conditions, S_2/S_1 ratios were 0.99±0.04 (n=6) and 0.96±0.02 (n=4) in human and rat detrusor samples, respectively.

Measurement of adenosine release

The procedures used to measure the release of adenine nucleosides (adenosine and inosine) were previously described (see *e.g.* Correia-de-Sá et al., 2006; Vieira et al., 2014). Experiments were performed in isolated rat detrusor strips without the mucosa using and automated perfusion system for sample collecting for given time periods, therefore improving the efficacy of HPLC (with diode array detection). After a 30-min equilibration period, the preparations were incubated with 2.8 mL gassed Tyrode's solution, which was automatically changed every 15 min by emptying and refilling the organ bath with the solution in use. In these experiments, samples retained for analysis were collected 15, 30 and 45 min after starting the experimental protocol (zero time). Isoprenaline (1 μM) or mirabegron (0.1 μM) were added to the incubation solution after the first collection (Basal, 15 min) and were kept in contract with the preparations at least for 15 min (Drug, 30 min) before starting electrical field stimulation (Drug + EFS, 45 min), which consisted in 3000 square wave pulses of 1 ms duration delivered at a 10-Hz frequency. Bath aliquots

(50-250 µl) were frozen in liquid nitrogen immediately after collection, stored at -20ºC (the enzymes are stable for at least 4 weeks) and analyzed within 1 week of collection by HPLC with diode array detection (Finnigan Thermo Fisher Scientific System LC/DAD, equipped with an Accela Pump coupled to an Accela Autosample, a diode array detector and an Accela PDA running the X-Calibur software chromatography manager). Chromatographic separation was carried out through a Hypersil GOLD C18 column (5 µM, 2.1 mm x 150 mm) equipped with a guard column (5 µm, 2.1 mm x 1 mm) using an elution gradient composed of ammonium acetate (5 mM, with a pH of 6 adjusted with acetic acid) and methanol. During the procedure the flow rate was set at 200 µl per min and the column temperature was maintained at 20ºC. The autosampler was set at 4ºC and 50 µl of standard or sample solution was injected, in duplicate, for each HPLC analysis. In order to obtain chromatograms and quantitative analysis with maximal sensibility, the diode array detection wavelength was set at 259 nm for adenosine and 248 nm for inosine.

Kinetics of cyclic AMP extracellular catabolism by HPLC

For the kinetic experiments of the extracellular catabolism of cyclic AMP and adenosine formation, human and rat detrusor strips without the mucosa were mounted in a 2-ml organ bath. All experiments were performed at 37ºC. Preparations were superfused with gassed (95% O_2 and 5% CO_2) Tyrode's solution. After equilibrium, the preparations were incubated with 30 µM of cyclic AMP (zero time). Samples of 75 µl were collected from the organ bath at different times up to 45 min for HPLC (with UV detection) analysis (LaChrome Elite, Merck, Germany) of the variation of substrate disappearance and product formation (Correia-de-Sá et al., 2006; Duarte-Araújo et al., 2004; Silva-Ramos et al., 2015a; Vieira et al., 2014). The stoichiometry of cyclic AMP conversion into their metabolites was kept unaltered (30 µM).

Immunofluorescence staining and confocal microscopy observation

Human and rat detrusor strips without the mucosa were stretched in all directions and pinned onto a Petri dish coated with Sygard®. The strips were then fixed in PLP solution (paraformaldehyde 2%, lysine 0.075 M, sodium phosphate 0.037 M, sodium periodate 0.01 M) for 16 h at 4ºC. Sixteen-micron sections were incubated with selected primary antibodies (table 9) diluted in an incubation buffer

(foetal bovine serum 5%, serum albumin 1%, Triton X-100 0.3% in PBS), at 4ºC, for 16 h. For double immunostaining, antibodies were combined before application to tissue samples. After washing away unbound primary antibody, the sections were incubated with secondary antibodies (table 9) in the dark for 2 hours, at room temperature. Negative controls were carried out by replacing the primary antibodies with non-immune serum; cross-reactivity for the secondary antibodies was tested in control experiments in which primary antibodies were omitted. Finally, tissue samples were mounted on optical-quality glass slides using VectaShield as antifade mounting media (VectorLabs) and stored in the dark at 4ºC. Observations were performed and analyzed with a laser-scanning confocal microscope (Olympus FluoView, FV1000, Tokyo, Japan).

Table 9. Primary and secondary antibodies used to stain human detrusor strips.

Antigen	Code	Host	Dilution	Supplier
Primary antibodies:				
Anti-VAChT	SAB5200240	Mouse	1:50	Sigma
Anti-A_1 receptor	Ab75177	Rabbit	1:250	Abcam
Anti-β_3 receptor	AAR-017	Rabbit	1:50	Alomone
Anti-β_3 receptor	MC-4198	Rabbit	1:50	MBL International
Anti-β_3 receptor	SC-1473	Goat	1:200	Santa Cruz
Anti-ENT1	ANT-051	Rabbit	1:50	Alomone
Anti-ENT2	ANT-052	Rabbit	1:50	Alomone
Anti-β-tubulin	Ab6046	Rabbit	1:2500	Abcam
Secondary antibodies:				
Alexa Fluor 488 anti-rabbit	A-21206	Donkey	1:1000	Molecular Probes®
Alexa Fluor 633 anti-ms	A-21050	Goat	1:1000	Molecular Probes
Alexa Fluor 568 anti-gt	A11057	Donkey	1:1000	Molecular Probes
IgG HRP anti-rb	Ab98503	Donkey	1:70000	Abcam
IgG HRP anti-gt	Ab97120	Donkey	1:25000	Abcam

Western blot analysis

Human and rat detrusor strips without the mucosa were homogenized in radio-immunoprecipitation assay buffer (Tris-HCl 25 mM (pH 7.6), NaCl 150 mM, sodium deoxycholate 1%, Triton-X-100 1%, sodium dodecyl sulfate (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, USA). 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 70ºC for 10 min, subjected to electrophoresis in 10% SDS-polyacrylamide gels and electrotransferred onto polyvinylidene difluoride (PVDF) membranes (MilliPore, MA, USA). Protein loads were 75 and 150 µg. The membranes were, then blocked in Tris-buffered saline (in mM: Tris-HCl 10 (pH 7.6), NaCl 150) containing Tween-20 0.05% and bovine serum albumin (BSA) 5% and, subsequently, incubated with two distinct anti-β_3 adrenoceptor primary antibodies either from MBL International Corporation (host species: rabbit; 1:200, MC-4198, also known as LS-A4198, Woburn MA, USA) or from Santa Cruz (host species: goat, 1:200, SC-1473, Dallas TX, USA) in the above blocking buffer overnight at 4ºC. Membranes were washed three times for 10 min in TBS/Tween-20 and incubated with horseradish peroxidase-conjugated secondary antibody (1:25000; Abcam, Cambridge, UK) for 1 h at room temperature. For normalization purpose, membranes were incubated with the mouse anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12) primary antibody from Santa Cruz (1:200, SC-32233, Dallas, TX, USA) following the procedures described above. Membranes were washed three times for 10 min 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, USA).

In vivo cystometry recordings

The experiments were carried out in urethane-anaesthetized rats (1.0-1.2 g/kg), spontaneously breathing. Core body temperature was kept between 36ºC and 38ºC with the help of a heating pad controlled by a thermosensor connected to a rectal probe. A catheter connected to an injection pump was inserted into the left jugular vein to permit saline infusion (4 ml/h/kg) and intravenous drugs application. After exposing the urinary bladder through a median abdominal incision, a three-barrel catheter was inserted through its dome. One barrel, was connected to an automate perfusion pump for saline and/or drugs infusion; a second barrel, was attached to a pressure transducer for continuous monitoring of intravesical pressure; the third barrel was used either to drain or to close the bladder circuit in order to initiate the micturition reflex. The bladder pressure was continuously monitoring of intravesical pressure; a second barrel, was attached to a pressure transducer for continuous monitoring of intravesical pressure; the third barrel was used either to

drain or to close the bladder circuit in order to initiate the micturition reflex. The bladder pressure was continuously monitored on a computer screen with a PowerLab data acquisition system (Chart 5, version 4.2 software; AD Instruments, USA), which was also used to record hemodynamic and respiratory parameters in the anaesthetized rat. After surgical preparation, a 60-min equilibration period was undertaken during which saline was infused into the urinary bladder at 0.04 ml/min and allowed to freely drain out of the bladder (open circuit). The micturition reflex was initiated by closing the draining barrel while keeping intravesical infusion of saline at a constant flow rate (0.04 ml/min), which is within the range used in previous studies to obtained stable micturition cycles during continuous cystometrograms in anaesthetized rat (Carneiro et al., 2014; Honda et al., 2012; Timóteo et al., 2014). The flow rate was 2 to 4-fold higher than the normal urinary debit in experimental rats (15-30 ml/d) and compared to the conditions used in standard filling cystometry (urodynamic test) in humans. Voiding contractions were assumed as large-amplitude rhythmic bladder contractions accompanied by urine draining through the urethra when bladder pressure reached a certain threshold. The intercontraction interval (ICI, min) and the pressure threshold (PTh, cm of H2O) that is required to initiate the voiding reflex are normally associated with the sensitive component of the micturition reflex (filling phase); conversely, the amplitude (A, cm of H_2O) and the duration (Δt, sec) of the voiding contractions are mostly associated with the motor component of the micturition reflex (emptying phase). For the sake of clarity, the results presented in this study will consider the percent variation of ICI values as compared to the control situation achieved after six consecutive voiding contractions of similar amplitude. Test drugs were applied either into the bladder lumen (by changing the syringue connected to the automate perfusion pump, 0.04 ml/min) or intravenously through the catheter inserted into the left jugular vein.

Drugs and Solutions

Adenosine 3',5'-cyclic monophosphate sodium salt monohydrate (3',5'cyclic AMP), hemicholinium-3, 1.3-dipropyl-8-cyclopentylxanthine (DPCPX), choline chloride, paraformaldehyde (prills), lysine, sodium periodate, anhydrous glycerol, fetal bovine, fenoterol hydrobromide, (±)-1-[2,3-(dihydro-7-methyl-1H-inden-4-yl]-3-[(1-methylethyl)amino]-2-butanol hydrochloride (ICI 118,551), 7β-acetoxy-8,13-epoxy-1α,6β,9α-trihydroxylabd-14-en-11-one (Forskolin, FSK), S-(p-nitrobenzyl)-6-

thioinosine (NBTI) were obtained from Sigma (St Louis, MO, USA); isoproterenol hydrochloride (Isoprenaline), 5-[(2R)-2-[[(2R)-2-(3-chlorophenyl)-2-hydroxyethyl]amino]propyl]-1,3-benzodioxole-2,2-dicarboxylic acid disodium salt (CL316,230A), 1-(2-ethylphenoxy)-3-[[(1S)-1,2,3,4-tetrahydro-1-naphthalenyl]amino]-(2S)-2-propanol hydrochloride (SR59230A hydrochloride), N^G-nitro-L-arginine methyl ester (L-NAME) hydrochloride, 4-(3-(cyclopentylox)-4-methoxyphenyl)pyrrolidin-2-one (Rolipram) were obtained from Tocris Bioscience (Bristol, UK); N-[[3-[(2S)-2-Hydroxy-3-[[2-[4-[(phenylsulfonyl)amino]phenyl]ethyl]amino]propoxy]phenyl] methyl]-acetamine (L-748,337) was obtained from Santa Cruz (Dallas, USA); mirabegron was obtained from Selleckchem.com (Houston, USA); dipyridamole was obtained from Boehringer Ingelheim (Germany); [methyl-^{3}H] choline chloride (ethanol solution, 80.6 Ci mmol^{-1}) was obtained from PerkinElmer (Boston, USA); serum albumin, Triton X-100 were obtained from Merck (Darmstadt, Germany). DPCPX was dissolved in a 5 mM stock solution in 99 % dimethylsulfoxide (DMSO) + 1% NaOH 1 M (v/v). Mirabegron, FSK and NBTI were dissolved in DMSO, respectively. Other drugs were prepared in Tyrode's solution. All stock solutions were stored as frozen aliquots at -20ºC. Dilutions of these stock solutions were made daily and appropriate solvent controls were done. No statistical 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 ± SD, 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 was carried out using Graph Pad Prism 6.04 for Windows software (La Jolla, USA). Paired and unpaired Student's t-test with Welch's correction was used for statistical analysis when parametric data was considered. One-way analysis of variance (ANOVA) followed by the Dunnett's post test was used for multiple comparisons. $P<0.05$ (two-tailed) values were considered statistically significant.

RESULTS

Immunolocalization of β_3-adrenoceptors and adenosine A_1 receptors in the human detrusor

The presence of β_3-adrenoceptors in the human detrusor was demonstrated by immunofluorescence confocal microscopy using three distinct commercially available antibodies (Figure 20A). The antibody from Alomone Labs (AAR-007) is directed against a 13 amino acid peptide if the second extracellular loop of the mouse β_3-adrenoceptor, which is 12/13 and 11/13 identical in the rat and human receptors, respectively. The antibody from MBL International Corporation (MC-4198, also known as LS-A4198) was raised against a synthetic 20 amino acid peptide from the N-terminal domain of the human β_3-adrenoceptor, which showed no homology with any other human protein as determined by BLAST (Basic Local Alignment Search Tool) analysis. The MC-4198 antibody was successfully been used to detect β_3-adrenoceptors by immunohistochemistry in human (Limberg et al., 2010) and rat (Kullmann et al., 2009) bladders. The third antibody from Santa Cruz Biotecnology Inc (SC-1472) was raised against a peptide mapping the C-terminus of the human β_3-adrenoceptor. Concordant staining of human tissues with two or more antibodies (e.g. MC-4198 and SC-1472) has been proposed to provide the most convincing evidence for β_3-adrenoceptor labeling in immunohistochemistry studies (Cernecka et al., 2012).

Our data show that β_3-adrenoceptors are expressed on smooth muscle fibers of the human (Figure 20A) and rat (Figure 20B) detrusor, although staining was also observed in cells located in the urothelium and sub-urothelial layer of the human bladder (Figure 20A, bottom micrographs) (*cf.* Kullmann et al., 2009; Limberg et al., 2010; Otsuka et al., 2008). In contrast to the predominant diffuse muscular pattern of the β_3-adrenoceptor immunostaining obtained concordantly with all tested antibodies (AAR-017, MC-4198 and SC-1472), the adenosine receptor co-localizes with the vesicular ACh transporter (VAChT) on bladder cholinergic nerve terminals (*cf.* Silva-Ramos et al., 2015a).

Using higher magnification images (right hand-side panels) we failed to co-localize β_3-adrenoceptors with VAChT immunostaining, thus suggesting that these receptors are not expressed on cholinergic nerve terminals (see also Otsuka et al.,

2013). The presence of adenosine A_1 receptor immunoreactivity was also abundantly detected in the urothelium of the human bladder (Figure 20A, bottom micrographs).

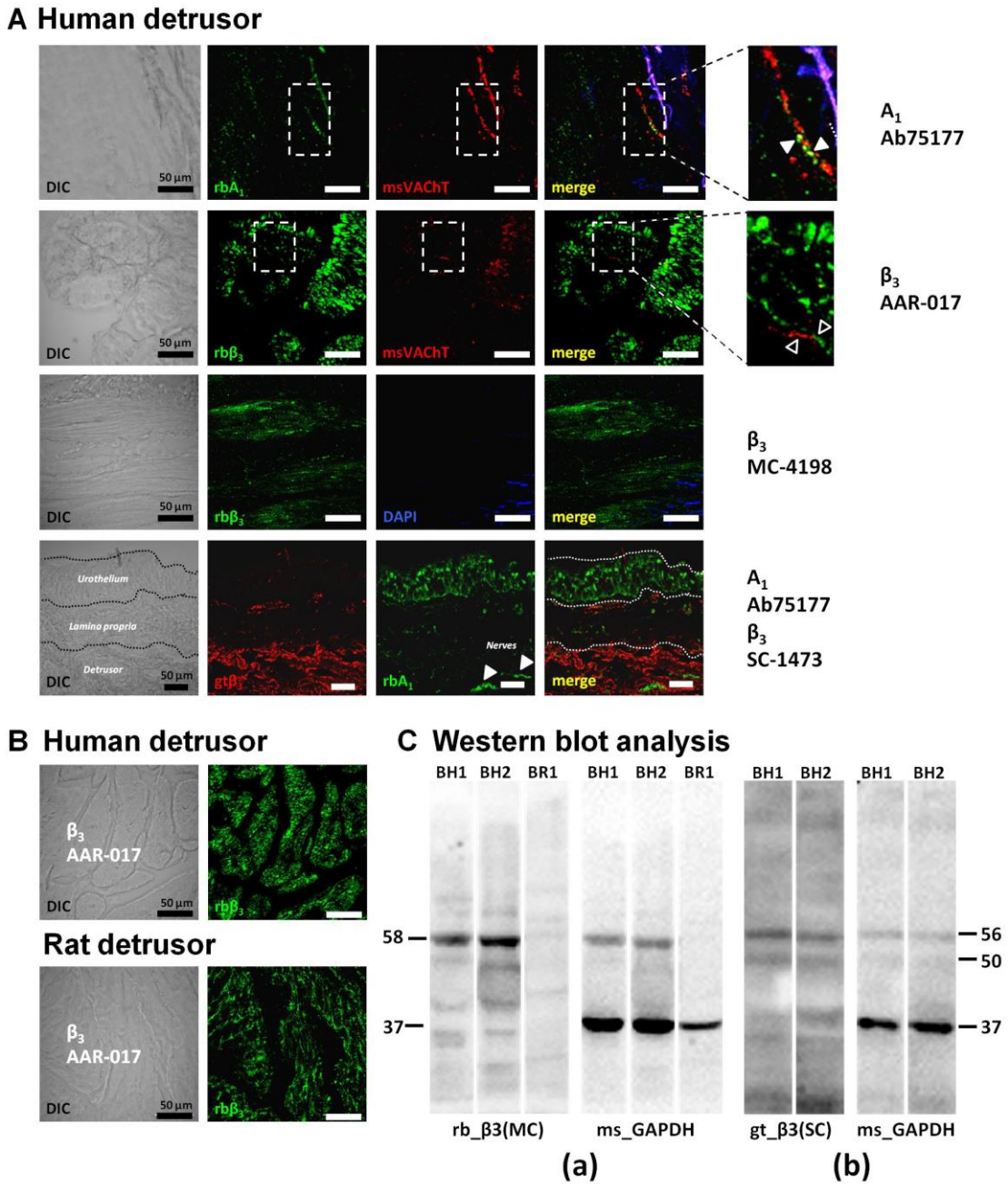


Figure 20. (A) Immunolocalization of β_3 and A_1 receptors in transverse sections of the human detrusor by confocal microscopy. Three distinct β_3 antibodies, AAR-017, MC-4198 and SC-1473, were used as indicated. A similar staining pattern was obtained for all antibodies showing that β_3-adrenoceptors are diffusely distributed among smooth muscle fibers, but are no present on cholinergic nerve fibers stained with the vesicular ACh transporter (VAChT); this aspect is indicated by the open arrowheads in the higher magnification image shown on the right hand-side panel. The uppermost panel shows that the adenosine A_1 receptor is predominantly expressed on VAChT-positive cholinergic nerve terminals of the human detrusor; co-localization of A_1 (green) and VAChT (red)

immunofluorescence is identified by the yellow staining (closed arrowheads) in the high magnification image shown in the right hand-side panel. Nuclei are stained with DAPI (blue). Purple staining corresponds to collagen autofluorescence emitting in all channels, including the 543 nm laser channel (with no secondary antibody, see *e.g.* Otsuka et al., 2008). Low magnification images to include the urothelium and suburothelial layers are shown in the bottom panel. **(B)** Comparison of the immunofluorescence staining pattern of transverse sections of the human and rat detrusor using the anti-β_3 antibody from Alomone (AAR-017). Images are representative or four different individuals. Differential interference contrast (DIC) images are shown for comparison. Scale bar = 50 µm. **(C)** Shown are representative immunoblots of the β_3-adrenoceptor in detrusor homogenates of two different male human subjects (BH1 and BH2) and one rat (BR1) run in parallel using antibodies directed to epitopes localized in the N- (MC-4198) and C- (SC-1473) terminus of the human β_3-adrenoceptor. Please note that human detrusor homogenates express a prominent band at 56-59 kDa that is compatible with the predicted size of a glycosylated form of the β_3-adrenoceptor; no band was detected with the MC-4198 in rat detrusor homogenates, since this antibody was specifically raised against the human β_3-adrenoceptor. The highly conserved GAPDH enzyme (EC 1.2.1.12, MW~37 kDa) was used as a reference protein. Gels were loaded with 150 µg or protein. Host species for antibody production were rabbit (rb), mouse (ms) and goat (gt).

Moreover, western blot analysis confirmed that the human detrusor expresses the β_3-adrenoceptor given to the fact that both SC-1472 and MC-4198 antibodies detected a major band with an apparent size of 56-59 kDa that is compatible with a glycosylated from of the human β_3-adrenoceptor (Figure 20C). Weaker bands, including one at the theoretical molecular mass (~44kDa) of the β_3-adrenoceptor based on its amino acid content, were also found in human, but not in rat, detrusor homogenates (Figure 20C). The lack of cross reactivity with the rat detrusor was anticipated as both, SC-1472 and MC-4198, antibodies were raised specifically against the human β_3-adrenoceptor. Data agree with previous findings using CHO stably transfected to express the human β_3-adrenoceptor, where SC-1472 was one of the most convincing antibodies to use in immunoblotting tests (Cernecka et al., 2012).

Stimulation of β_3-adrenoceptors in human and rat urinary bladders decrease evoked [^{3}H]ACh release depending on the activation of inhibitory adenosine A_1 receptors

Figure 21A shows that the β-adrenoceptor agonist, isoprenaline (1 µM), decreased by 47 ± 5% (n=6) the release of [^{3}H]ACh evoked by electrical field

stimulation (10 Hz, 200 pulses) of the human detrusor; these results are in agreement with the study of D'Agostino et al. (D'Agostino et al., 2015). The inhibitory effect of isoprenaline (1 μM) was significantly ($P<0.01$) attenuated by the β_3-adrenoceptor antagonist, SR59230A hydrochloride (100 nM, Figure 21B) (Jedlitschky et al., 2000), wich exhibits high affinity (pK_i 6.9-8.4) for the human β_3 receptor subtype although it can also bind to other β-adrenoceptor subtypes. Inhibition of isoprenaline relaxation responses by SR59230A in the human bladder has been reported before (Takeda et al., 1999). Likewise, mirabegron, used in a concentration (0.1 μM) that selectively activates β_3-adrenoceptors in the human bladder (Takasu et al., 2007), mimicked the inhibitory effect of isoprenaline (1 μM); mirabegron (0.1 μM) decreased electrically-evoked [^{3}H]ACh release by 57±6% (n=7, Figure 21C). The inhibitory effect of mirabegron (0.1 μM) was significantly ($P<0.01$) attenuated by L748,337 (30 nM), a more selective β_3-adrenoceptor antagonist (K_i 4 nM) than SR59320A hydrochloride (Candelore et al., 1999).

Taking into consideration that β_3-adrenoceptors are not present on cholinergic nerve terminals of the human bladder (see Figure 20), yet both isoprenaline and mirabegron were one to two log orders of magnitude more potent in decreasing [^{3}H]ACh release than in reducing nerve-evoked contractions of the human detrusor (D'Agostino et al., 2015, reviewed in Michel and Korstanje, 2016), we hypothesized the involvement of a retrograde messenger like adenosine bolstering inhibition of evoked transmitter release via prejunctional A_1 receptors (Silva-Ramos et al., 2015a). Figure 21 shows that transmitter release inhibition caused by isoprenaline (1 μM, Figure 21B) and mirabegron (0.1 μM, Figure 21C) was prevented ($P<0.01$) by the selective adenosine A_1 receptor antagonist, 1,3-dipropyl-8-cyclopentylxantine (DPCPX, 100 nM).

Likewise, isoprenaline (1 μM) decreased by 38 ± 1% (n=4) the evoked [^{3}H]ACh release in rat urinary bladder strips without the mucosa and its inhibitory effect was also prevented by the selective blockade of β_3-adrenoceptors and adenosine A_1 receptors with SR59230A (100 nM, Figure 22A) and DPCPX (100 nM, Figure 22C), respectively, whereas the β_2-adrenoceptor antagonist, ICI118,551 (50 nM), was devoid of effect (Figure 22A). Moreover, the isoprenaline (1 μM) inhibitory action on evoked [^{3}H]ACh outflow was mimicked by the "rodent selective" β_3-adrenoceptor agonist, CL316,243 (1 μM) (Evans et al., 1999), whereas transmitter

release from rat detrusor strips was insensitive to fenoterol (50 nM, a β_2-adrenoceptor agonist) (Figure 22B).

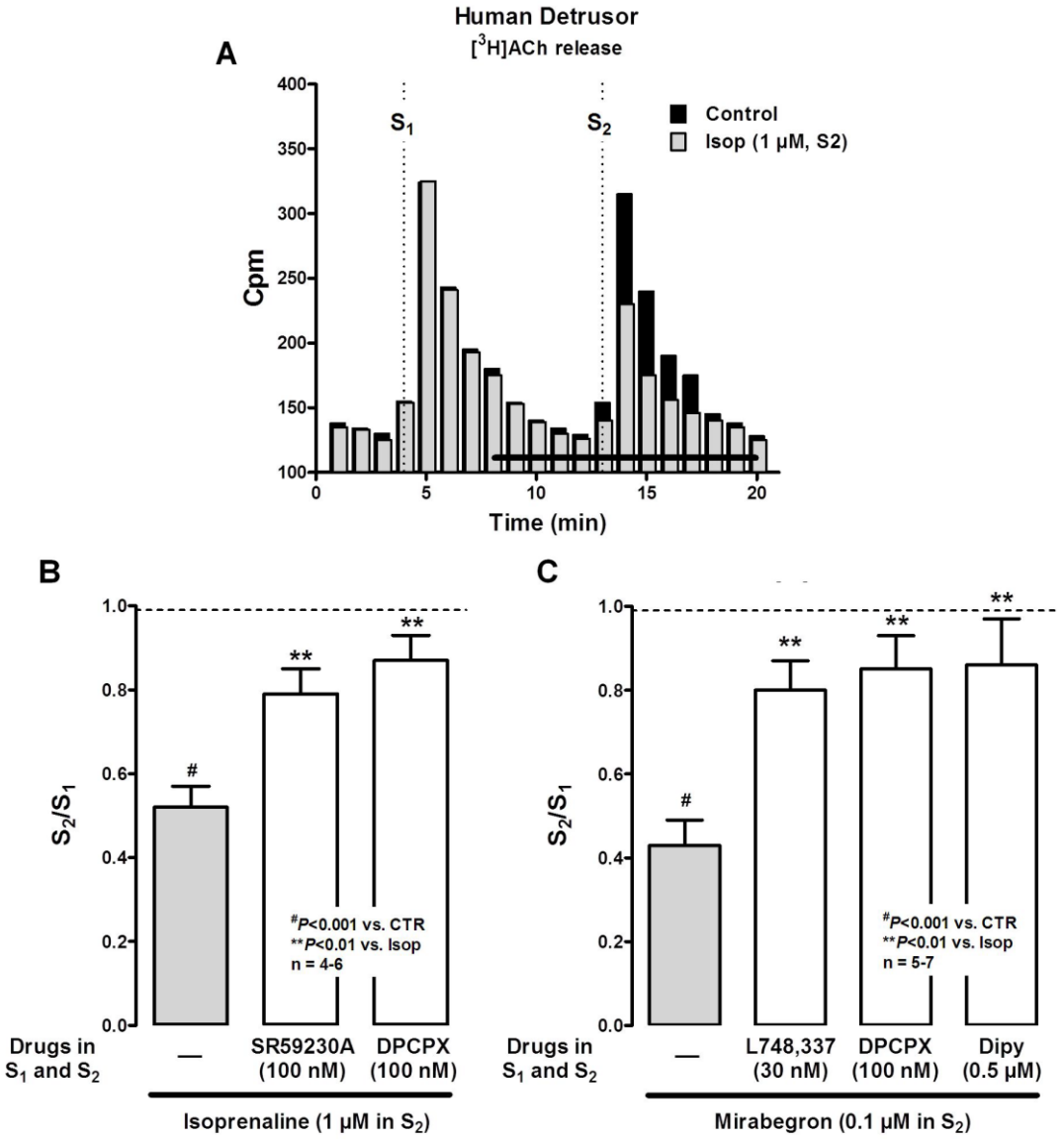


Figure 21. (A) Inhibitory effect of isoprenaline on electrically-evoked [^{3}H]ACh release from urothelium-denuded human detrusor strips. Ordinates represent tritium outflow expressed in scintillations per min (cpm). Abscissa indicates the times at which samples were collected. [^{3}H]ACh release was elicited by electrical field stimulation (10 Hz, 200 pulses of 0.2 ms duration) twice, starting at 4th (S_1) and 13th (S_2) minutes after the end of washout (zero time). Isoprenaline (1 µM, grey bars) was added to the incubation media 6 min before S_2 (horizontal bar). Bottom panels show the inhibitory effects of isoprenaline (1 µM, **B**) and mirabegron (0.1 µM, **C**) on [^{3}H]ACh release from electrically stimulated human detrusor strips in the absence and in the presence of selective β_3-receptor antagonists, SR59230A (100 nM, **B**) and L748,337 (30 nM, **C**), and of the A_1 receptor antagonist, DPCPX (100 nM, **B and C**); the effect of mirabegron in the presence of dipyridamole (Dipy, 0.5 µM) is also shown in **panel C**. Receptor antagonists and Dipy were present throughout the assay, including S_1 and S_2. The ordinates are changes in S_2/S_1 ratios compared to the S_2/S_1 ratio obtained without

addition of any drug (dotted horizontal line). The data are means ± SD of three to six individuals; duplicates were performed for each individual experiment. #*P*<0.001 (one-way ANOVA followed by the Dunnett's post test) represent significant differences when compared to the control situation; ***P**<0.01 (one-way ANOVA followed by the Dunnett's post test) represent significant differences when compared to the inhibitory effect of isoprenaline.

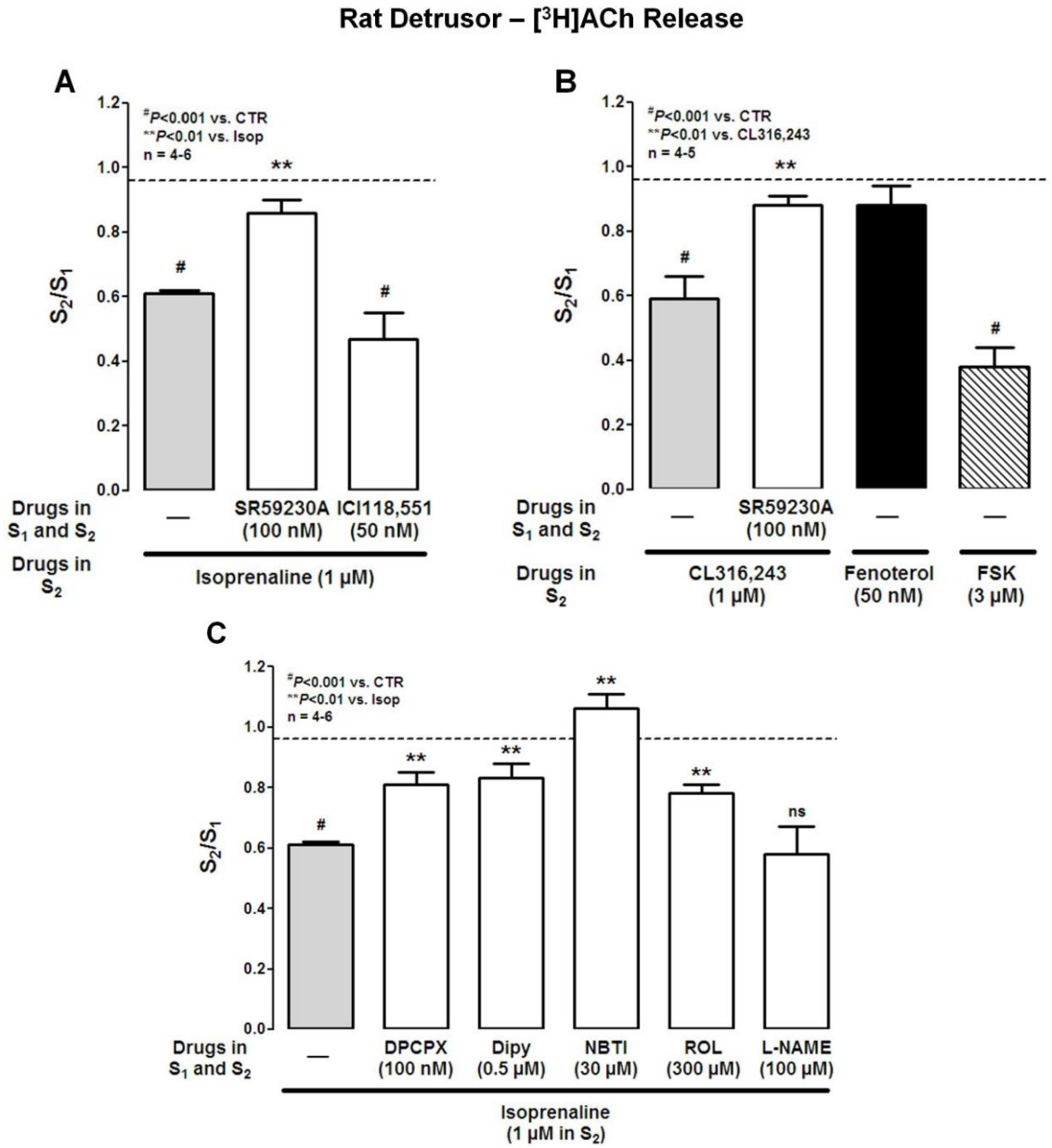


Figure 22. Changes in the inhibitory effect of isoprenaline on electrically-evoked [^{3}H]ACh release from urothelium-denuded rat detrusor strips in the presence of SR59230A (100 nM, β_3-adrenoceptor antagonist), ICI118,551 (50 nM, β_2-adrenoceptor antagonist) (**panel A**), DPCPX (100 nM, adenosine A_1 receptor antagonist), dipyridamole (Dipy, 0.5 µM, ENT1 and ENT2 inhibitor), NBTI (30 µM, selective ENT1 inhibitor), rolipram (ROL, 300 µM, phosphodiesterase IV inhibitor) and L-NAME (100 µM, NO synthase inhibitor) (**panel C**). For comparison purposes we also tested the effect

of selective β_3 (CL316,243, 1 μM), and β_2 (fenoterol, 50 nM) adrenoceptor agonists and the adenylyl cyclase activator, forskolin (FSK, 3 μM), on evoked [^{3}H]ACh release from rat detrusor strips under similar experimental conditions (**panel B**). [^{3}H]ACh release was elicited by electrical field stimulation (10 Hz, 200 pulses of 0.2 ms duration) twice, starting at 4th (S_1) and 13th (S_2) minutes after the end of washout (zero time). Isoprenaline, CL316,243, fenoterol and forskolin were added to the incubation media 6 min before S_2; SR59230A, ICI118,551, DPCPX, Dipy, NBTI, ROL, and L-NAME were present throughout the assay, including S_1 and S_2. Ordinates are changes in S_2/S_1 ratios compared to the S_2/S_1 ratio obtained without addition of any drug (dotted horizontal line). The data are means ± SD of an n number of individuals; duplicates were performed for each individual experiment. $^{\#}P<0.001$ (one-way ANOVA followed by the Dunnett's post test) represent significant differences when compared to the control situation; $^{**}P<0.01$ (one-way ANOVA followed by the Dunnett's post test) represent significant differences when compared to the inhibitory effect of isoprenaline or CL316,243.

Based on equipotency of CL316,243 and isoprenaline it has been proposed that rat bladder relaxation predominantly occurs via the β_3-adrenoceptor subtype (Takeda et al., 2000; Woods et al., 2001; Yamazaki et al., 1998). Involvement of β_3-adrenoceptors mediating inhibition of transmitter release caused by CL316,243 (1 μM) was further confirmed by showing that the effect of the β_3-receptor agonist was also abolished in the presence of SR59230A (100 nM) (Figure 22B).

The inhibitory role of β_3-adrenoceptors on evoked [^{3}H]ACh release from the urinary bladder requires adenosine release via equilibrative nucleoside transporters

β_3-adrenoceptors usually couple to adenylyl cyclase via G_s proteins to generate intracellular cyclic AMP. In this context, we show in Figure 22B that the inhibitory effects of isoprenaline (1 μM) and CL316,243 (1 μM) were reproduced by the adenylyl cyclase activator, forskolin (3 μM), which on its own decreased evoked [^{3}H]ACh release by 61±7% (n=5). In many tissues intracellular cyclic AMP accumulation translates into increased extracellular adenosine amounts, which negatively modulates ACh release from cholinergic bladder nerves (Silva-Ramos et al., 2015a). Therefore, we tested whether this mechanism was responsible for the β_3-adrenoreceptor inhibitory control of cholinergic neurotransmission in rat detrusor strips.

Cyclic AMP is effectively and rapidly degraded by phosphodiesterases into AMP and adenosine inside the cell, and the nucleoside may be subsequently

transported to the extracellular milieu via equilibrative transporters (ENT). The influence of this pathway was evaluated by treating rat detrusor strips prior to the application of isoprenaline (1 µM) with a phosphodiesterase IV inhibitor, rolipram (300 µM), and two equilibrative nucleoside transport (ENT) blockers, dipyridamole (0.5 µM) and S-(4-nitrobenzyl)-6-thioinosine (NBTI, 30 µM); in contrast to dipyridamole, NBTI is a selective ENT1 inhibitor with no affinity for phosphodiestesare enzymes. Results show that in the presence of rolipram (300 µM), dipyridamole (0.5 µM) or NBTI (30 µM), isoprenaline (1 µM) was decreased [^{3}H]ACh release induced by electrical stimulation of rat detrusor strips to a lesser extend compared to the control situation (Figure 22C). Likewise, dipyridamole (0.5 µM) also prevented the inhibitory effect of mirabegron (0.1 µM) on evoked [^{3}H]ACh release from isolated human detrusor strips (Figure 22C). Notwithstanding the fact that β-adrenoceptor-mediated release of NO has been observed in the rat urothelium (Birder et al., 2002) and NO-mediated responses to β_3-adrenoceptor agonists have been observed in rat aorta and human coronaries, isoprenaline-induced reduction of [^{3}H]ACh release from stimulated rat detrusor strips apparently does not involve NO because inhibition of NO synthase activity with L-NAME (100 µM) had not effect (Figure 22C) (*cf.* Michel and Korstanje, 2016). Figure 23 shows that incubation of human and rat detrusor strips for 15 min with mirabegron (0.1 µM, Figure 23A) and isoprenaline (1 µM, Figure 23B), respectively, increased significantly (P<0.05) the concentration of adenosine in the incubation fluid, while the amount of inosine remained fairly constant compared to basal conditions. Isoprenaline (1 µM)-induced adenosine outflow increased further (from 174 ± 21 nM to 241 ± 13 nM, n=4, P<0.0001) after electrical stimulation of rat detrusor strips (data not shown) and it was largely attenuated following preincubation with SR59230A (100 nM) or dipyridamole (0.5 µM) (Figure 23B). The extracellular concentration of adenosine measured under these experimental conditions is probably underestimated, because it was obtained in a rather large volume of incubation media (2.8 ml) compared with the reduced interstitial volume of the bladder *in vivo*.

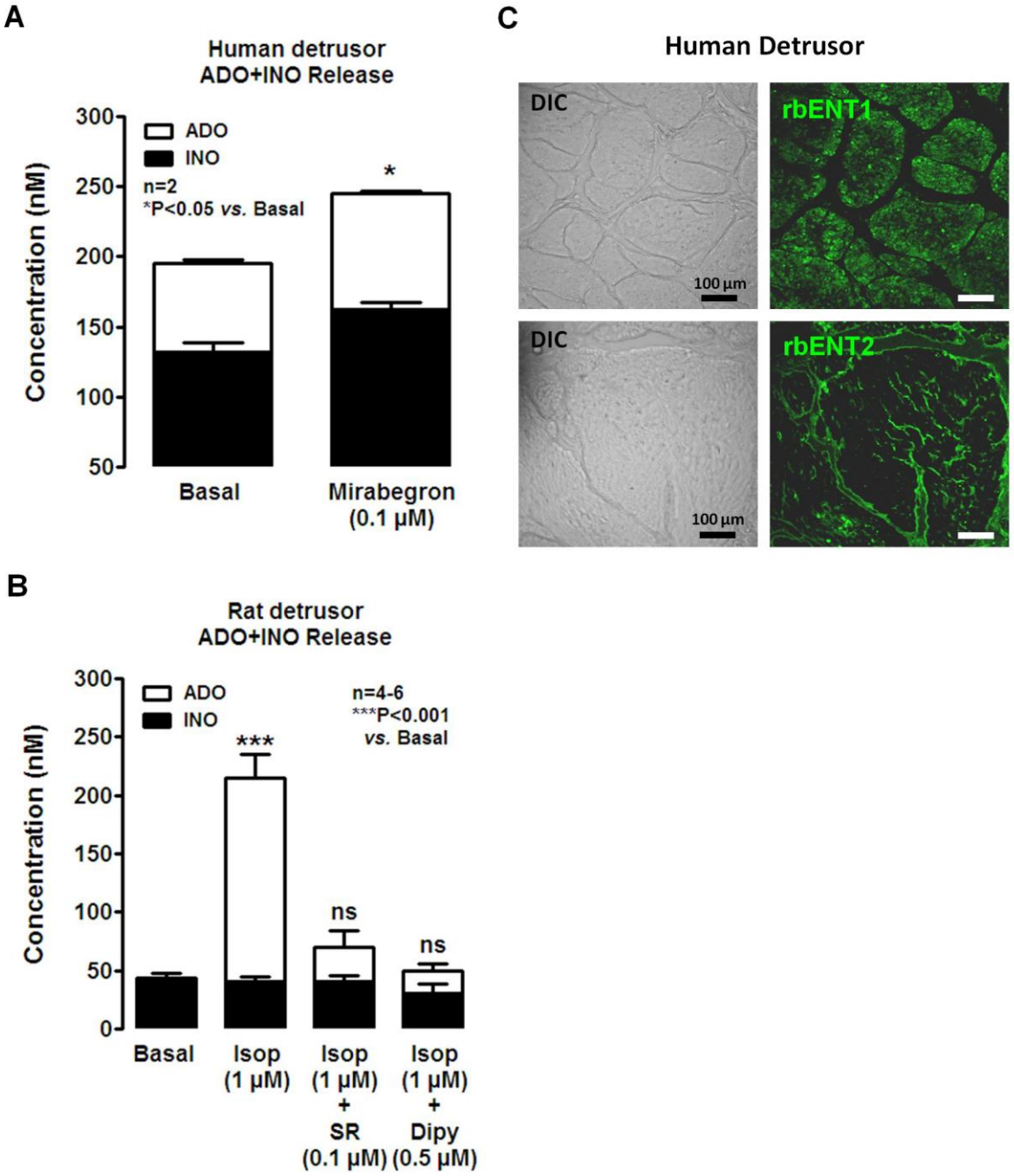


Figure 23. Mirabegron (0.1 µM, **A**) and isoprenaline (Isop, 1 µM, **B**) favour the release of adenosine from urothelium-denuned human and rat detrusor strips, respectively. Mirabegron and isoprenaline were allowed to contact the preparations for 15 min before sample collection. The ordinates represent the concentration of adenosine (ADO, white bars) and inosine (INO, black bars) detected by HPLC with diodo array detection in samples collected from the incubation media at 15 min intervals (for details, see Materials and Methods). The data are means ± SD of two man and four rats, respectively; duplicates were performed for each individual experiment. $P<0.05$ and $^{***}P<0.001$ (one-way ANOVA followed by the Dunnett's post test) represent significant differences when compared to the control situation (Basal). Please note that the release of adenosine returned to control levels when isoprenaline (1 µM) was applied together with SR59230A (0.1 µM) or dipyridamole (Dipy, 0.5 µM). (**C**)

Confocal micrographs showing the immunoreactivity against equilibrative nucleoside transporters, ENT1 and ENT2, in transverse sections of human detrusor strips. ENT1 (upper panel) and ENT2 (bottom panel) immunoreactivity is shown in green; differential interference contrast (DIC) images are also shown. Images are representative or four individuals. Scale bars = 100 µm.

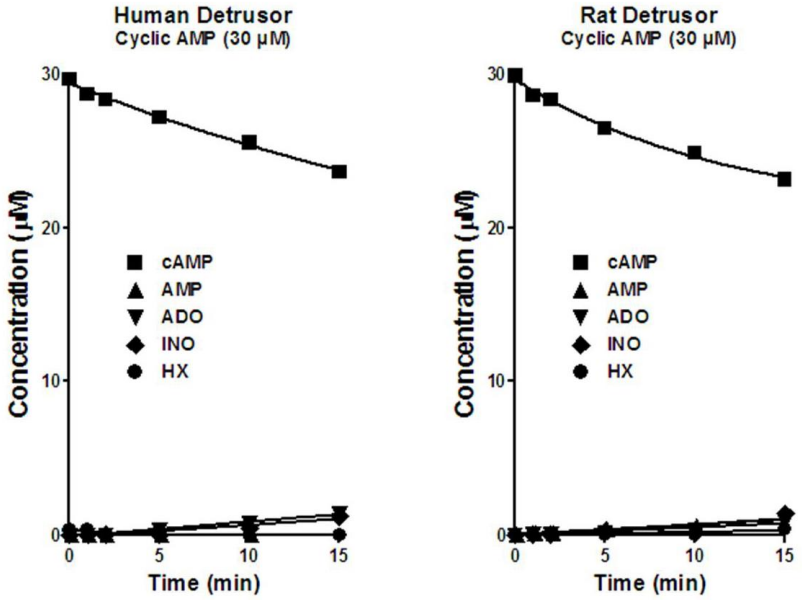


Figure 24. Kinetics of the extracellular catabolism of cyclic AMP in urothelium-denuded detrusor strips from human and rat bladders. Cyclic AMP (30 µM) was added at zero time to the incubation medium. Samples were collected from the incubation fluid at the indicated times on the abscissa and analysed by HPLC with UV detection to quantify cyclic AMP (squares), AMP (upward triangles), adenosine (ADO, downward triangles), inosine (INO, lozenges) and hypoxanthine (HX, circles). Average results obtained from four individuals of each group made in triplicate; the vertical bars represent SD and are shown when they exceed the symbols in size.

In support for the involvement of equilibrative nucleoside transporters mediating adenosine outflow in response to β_3-adrenoceptors activation in the urinary bladder, we show in Figure 23C that the human detrusor exhibits significant immunoreactivity against NBTI-sensitive ENT1 (SLC29A1) and NBTI-insensitive ENT2 (SLC29A2) subtypes, which are localized predominantly on the plasma membrane of smooth muscle fibers and at interstitial spaces of detrusor muscle bundles, respectively.

Alternatively, cyclic AMP can be actively pumped out from many cell types including the rat detrusor (Uchida et al., 2005), thereby contributing to extracellular adenosine formation via an enzymatic cascade involving ecto-nucleotide

pyrophosphatase/phosphodiesterase 1 (ENPP1) and ecto-5'-nucleotidase/CD73 (see *e.g. ref.* Chiavegatti et al., 2008; Sassi et al., 2014). Figure 24 shows enzymatic kinetic experiments where we studied the time course of the extracellular catabolism of cyclic AMP and formation of its metabolites, including AMP, adenosine, inosine and hypoxanthine, in detrusor strips of both human and rat urinary bladder. The activity of ENPP1 calculated 15-min after cyclic AMP (30 µM) application the ratio of [AMP+ADO+INO+INO]:[cyclic AMP] per min was $7.5x10^{-3}$ (n=4) in the detrusor human, whereas under similar experimental conditions dephosphorylation of AMP (30 µM) by ecto-5'-nucleotidase/CD73 calculated by the ratio of [ADO+INO+INO]:[AMP] per min in a previous report from our group was significantly (P<0.05) higher ($24.4x10^{-3}$, n=4) (Silva-Ramos et al., 2015a). Accordingly, the amount of adenosine (plus inosine and hypoxanthine) in the incubation fluid originating from the extracellular catabolism of cyclic AMP (30 µM) in the human bladder did not surprass 2.62 µM, while this value reached 7.98 µM following 15-min incubation with AMP (30 µM). Similar results were obtained in the rat detrusor as can be appreciated in Figure 24.

Activation of β_3-adrenoceptors decreases non-voiding microcontractions and the micturition frequency in anaesthetized rats in vivo with the participation of adenosine A_1 receptors

Figure 25 shows that instillation of isoprenaline (0.1-1000 nM) into the lumen of the urinary bladder of anaesthetized rats decreased the number and amplitude of non-voiding spontaneous microcontractions and the micturition frequency in a concentration-dependent manner; the latter was evidenced by prolongation of the intercontraction interval (ICI) without significantly (P>0.05) modifying the amplitude (A) and the duration (Δt) of voiding bladder contractions (Figure 25A).The calculated pEC_{50} value for isoprenaline under the present experimental conditions (-8.84±0.37; E_{max} 42.03±4.53%; n=5) is consistent with reported values (-8.3 and -9.1) in the literature for β_3-adrenoceptors activation in the rat isolated detrusor (Yamazaki et al., 1998). In support of this theory, the selective β_3-adrenoceptor agonist, CL316,243 (pEC_{50} -8.61±0.43; E_{max} 36.90±4.60%; n=5), was equipotent to isoprenaline in the bladder of anaesthetized rats, whereas β_2-adrenoceptor activation with fenoterol was less effective (pEC_{50} -7.90±0.085; E_{max} 13.87±4.38%; n=5) (Figure 25Bi). The inhibitory effect of isoprenaline (0.1-1000 nM) on the micturition reflex seems to be

mediated by targets located in the urinary bladder, since instillation of this drug into the bladder lumen did not (P>0.05) affect heart rate in the anaesthetized rat which would have happened if the drug entered the systemic circulation or was applied via i.v. or i.p., *i.e.* less than 5% increase in heart rate compared to control (405±52 bpm, n=5) was observed when isoprenaline was used at the highest concentration. A similar result was obtained for the other β-adrenoceptor agonists.

Pretreatment with the selective β_3-receptor antagonist, SR59230A (100 nM), prevented the decrease in the voiding frequency caused both by isoprenaline (0.1-1000 nM) (Figure 25A and 25Bii) and CL316,243 (0.1-1000 nM) (Figure 25Biii), whereas blockade of the β_2-adrenoceptor with ICI228,552 (50 nM) had only a limited effect on the concentration-response curve of isoprenaline (0.1-1000 nM) (Figure 25 Bii). Inhibition of bladder activity detected with isoprenaline (0.1-1000 nM) was also prevented by selectively blocking adenosine A_1 receptors with DPCPX (100 nM) (Figure 25A and 25Biv) or by inhibiting adenosine outflow to the extracellular milieu via equilibrative nucleoside transporters (ENT) with dypiridamole (0.5 μM) or NBTI (30 μM) were active either when superfused into the bladder lumen together with isoprenaçine (Figure 25) or by continuous *i.v.* infusion (data not shown). Neither of these drugs affected the urodynamic parameters when applied alone, independently of the route or administration, even when the concentration of SR59320A was increased to 100 μM. These results suggest that the inhibitory effect of β_3-adrenoceptor activation on the distension-induced micturition reflex in the rat in vivo is under the tonic control of adenosine A_1 receptors activation, but saline bladder distension does not generate on its own sufficient amounts of extracellular endogenous ligands, such as adenosine and/or noradrenaline, to promote activation of A_1 and β_3 receptors, under the present experimental conditions.

Interestingly, the adenylyl cyclase activator, forskolin (0.01-10 μM), also mimicked the inhibitory effect of β_3 adrenoceptor activation in the bladder of anaesthetized rats. Forskolin concentration-dependently increased (pEC_{50} - 6.75±0.30; E_{max} 39.30±4.38%; n=5) the duration of ICI without any modification in the amplitude (A) and the duration (Δt) of voiding bladder contractions.

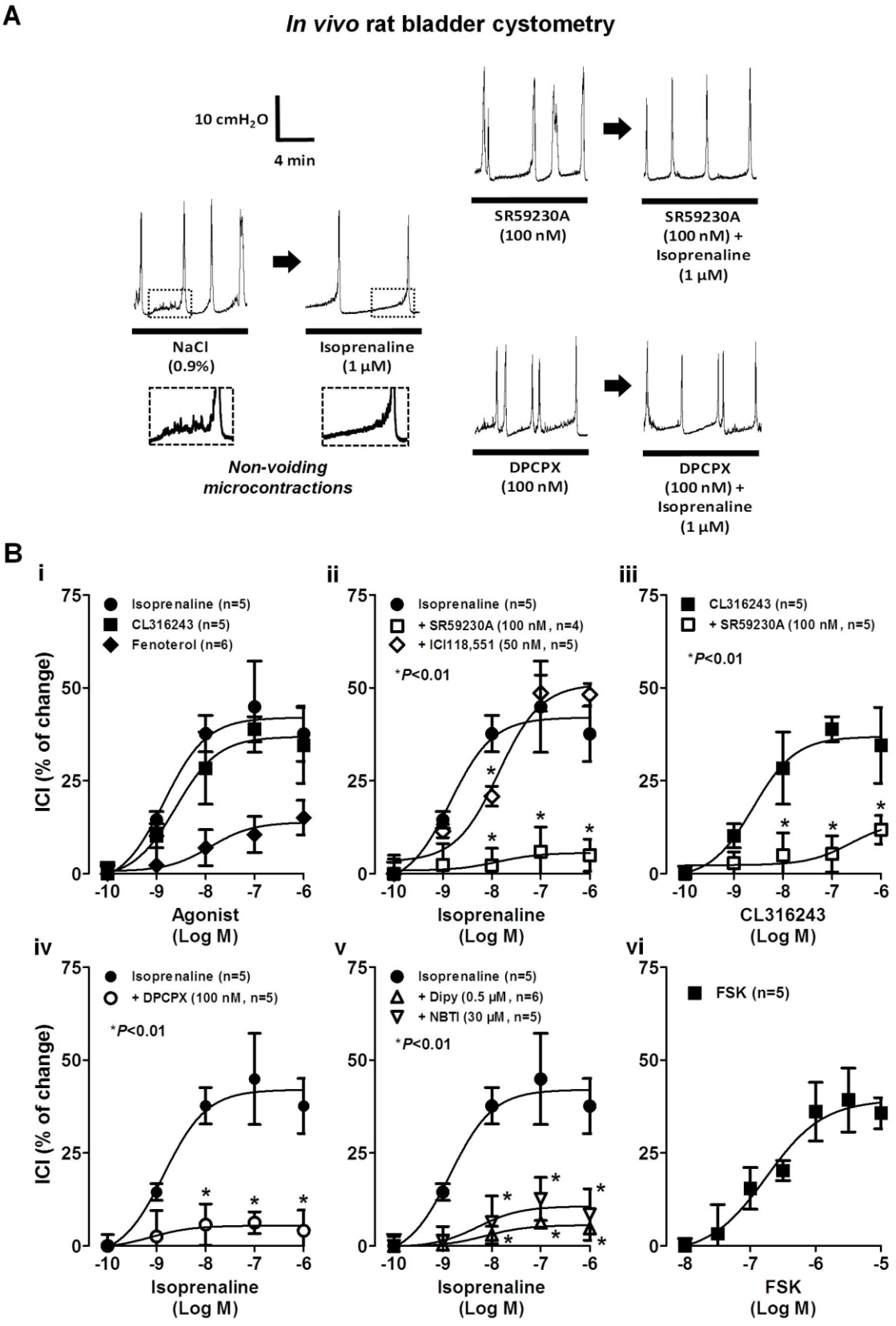

A
In vivo rat bladder cystometry
10 cmH2O
4 min
NaCl (0.9%)
Isoprenaline (1 µM)
Non-voiding microcontractions
SR59230A (100 nM)
SR59230A (100 nM) + Isoprenaline (1 µM)
DPCPX (100 nM)
DPCPX (100 nM) + Isoprenaline (1 µM)
B
i
Isoprenaline (n=5)
CL316243 (n=5)
Fenoterol (n=6)
ICI (% of change)
Agonist (Log M)
ii
Isoprenaline (n=5)
+ SR59230A (100 nM, n=4)
+ ICI118,551 (50 nM, n=5)
*P<0.01
Isoprenaline (Log M)
iii
CL316243 (n=5)
+ SR59230A (100 nM, n=5)
*P<0.01
CL316243 (Log M)
iv
Isoprenaline (n=5)
+ DPCPX (100 nM, n=5)
*P<0.01
ICI (% of change)
Isoprenaline (Log M)
v
Isoprenaline (n=5)
+ Dipy (0.5 µM, n=6)
+ NBTI (30 µM, n=5)
*P<0.01
Isoprenaline (Log M)
vi
FSK (n=5)
FSK (Log M)

Figure 25. (A) Bladder cystometry recording during normal saline (0.9% w/v of NaCl) and isoprenaline (1 μM) infusion into the urinary bladder of urethane-anaesthetized rats verified in the absence and in the presence of selective β_3- and A_1 receptor antagonist, SR59230A (100 nM) and DPCPX (100 nM), respectively. Large-amplitude bladder contractions correspond to voiding contractions when they were accompanied by urine draining through the urethra. Traces obtained during the filling phase of the micturition reflex (dotted rectangles) were enlarged to show non-voiding spontaneous microcontractions. Stable urodynamic responses to isoprenaline were reached in 10-15 min. **(B)** Show concentration-response curves of isoprenaline (0.1-1000 nM)-induced prolongation of the intercontraction interval (ICI) of voiding contractions in the absence and in presence of SR59230A (100 nM), ICI118,551 (50 nM), DPCPX (100 nM), dypiridamole (0.5 μM) and NBTI (30 μM); The effects of fenoterol (0.1-1000 nM, a β_2-adrenoceptor agonist), CL316,243 (0.1-1000 nM, a β_3-adrenoceptor) and forskolin (FSK, 0.01-10 μM, an adenylyl cyclase activator) are also shown for comparison. Control values correspond to zero percent variation. The vertical bars represent SD of an n number of animals (shown in parenthesis). $^*P<0.01$ (one-way ANOVA followed by the Dunnett's post test) represents significant differences as compared to the effect of isoprenaline or CL316,243 applied alone.

DISCUSSION

There is an increasing interest concerning the role of β_3-adrenoceptor agonists for the management of the OAB syndrome as an efficacious and more tolerable alternative to antimuscarinic drugs (Thiagamoorthy et al., 2015). It was first though that improvement of OAB symptoms by β_3-adrenoceptor agonists, like mirabegron and solabegron, were mostly due to direct relaxation of the detrusor smooth muscle (Igawa et al., 1999; Takeda et al., 1999; Wuest et al., 2009). However, newer theories suggest that β_3-adrenoceptor agonists decrease OAB symptoms also by reducing bladder afferent nerve activity (Woods et al., 2001) and/or ACh release from parasympathetic postganglionic nerves innervating the detrusor (D'Agostino et al., 2015). These new paradigms may be clinically relevant because, contrary to the initial belief that normally no parasympathetic nerve drive was observed during bladder storage, recent evidence demonstrate that there is release of ACh from both neuronal and non-neuronal sources, such as the urothelium and lamina propria, during the filling phase of the micturition cycle (Yoshida et al., 2004; reviewed in Winder et al., 2014). Moreover, this cholinergic trend may be directly or indirectly exaggerated in overactive bladder patients via the release of ATP and other "danger" molecules (Silva et al., 2015).

Notwithstanding the recent therapeutic advances towards the use of β_3-adrenoceptor agonists for lower urinary tract syndromes, research involving β_3-adrenoceptors has been hampered due to unique properties to the β_3-adrenoceptor which make extrapolation of findings between human and rodent models very difficult to reconcile owe to considerable species differences in the selectivity and potency of commonly used drugs. Here, we tested an array of the most selective agonists and antagonists available for human and rat β_3-adrenoceptors in comparison with the effect of the non-selective β-adrenoceptor agonist, isoprenaline. Regarding the involvement of β_3-adrenoceptor on [^{3}H]ACh release from stimulated detrusor strips our results show that the inhibitory effect of isoprenaline was mimicked by mirabegron and CL316,243, which exhibit some degree of selectivity for β_3-adrenoceptors in human and rat tissues, respectively, when applied in the submicromolar concentration range. It is worth noting that mirabegron is about 8-fold more potent in the human (Takasu et al., 2007) as compared to the rat bladder (Takasu et al., 2007; reviewed in Michel and Korstanje, 2016) and the opposite was observed concerning the selectivity of CL316,243 for rodent β_3-adrenoceptors (Evans et al., 1999). The effects of mirabegron and CL316,243 were prevented by blockage of β_3-adrenoceptors in human and rat detrusor with L748,337 and SR59230A, respectively. While SR59230A is regarded as a potent β_3-adrenoceptor antagonist in rodents, it can hardly distinguish the human β_3-adrenoceptors from other adrenoceptors (β_1 and β_2) expressed in the same tissue (Baker, 2010; Candelore et al., 1999). Mirabegron also mimicked the enhancing effect of isoprenaline on extracellular adenosine accumulation on human detrusor strips. Overall these results proof the involvement of β_3-adrenoceptors in the inhibitory control of cholinergic neurotransmission and bladder reactivity through the release of adenosine from the detrusor.

In this study, we demonstrated by western blot analysis and immunofluorescence confocal microscopy using three distinct commercial antibodies (AAR-017, MC-4198 and SC-1473) that β_3-adrenoceptors are localized on detrusor smooth muscle fibers (*cf.* Fujimura et al., 1999; Igawa et al., 1998; Igawa et al., 1999). These receptors seem to be absent from VAChT-positive cholinergic nerves of the human urinary bladder cells of intramural nerves (Limberg et al., 2010). Contradictory evidence for β_3-adrenoceptors being present on ACh-containing nerve fibers of the human detrusor exists considering the fact that since submission of the

present manuscript new information emerged (Coelho et al., 2017); using epifluorescence microscopy, Coelho et al. (2017) showed that β_3-adrenoceptors stained with the SC-1472 antibody co-localized with VAChT-positive nerve fibers in the human detrusor, but the density of nerve varicosities expressing the cholinergic marker far exceeded those expressing β_3-immunoreactivity. These findings challenge the current view about the mechanism and site of action of β_3-adrenoceptor agonists on cholinergic bladder nerves activity and open the door for novel targets (e.g. adenosine) indirectly mediating inhibition of neurogenic bladder contractions. Another puzzling finding that has been insufficiently explored so far is the higher effectiveness of β_3-adrenoceptor agonists in decreasing (D'Agostino et al., 2015; Gillespie et al., 2015; Rouget et al., 2014). This led several authors to suggest that in vivo concentrations of these drugs following clinical application are below the minimum required to affect detrusor myogenic contractions as a mechanism underlying relief of OAB symptoms (Andersson, 2016). Our results strongly suggest that β_3-adrenoceptors activation (either with isoprenaline or mirabegron) significantly increases adenosine release from detrusor smooth muscle fibers, via NBTI-sensitive equilibrative nucleoside transporters (ENT1), leading to retrograde activation of A_1 receptors and, thereby, to inhibition of [^{3}H]ACh release from cholinergic nerves of human and rat urinary bladders. Reduction of the cholinergic tone responsible for non-voiding bladder contractions, acting in coordination with the sensory inhibitory drive caused by β_3-adrenoceptors agonists and endogenous adenosine (via A_1 receptors), may increase bladder compliance during the filling phase of the micturition cycle resulting in improvement of OAB symptoms. This is an interesting feature that might put adenosine signaling pathways, consisting of metabolizing enzymes, nucleoside transporters, and membrane receptors, in the centre of research for novel therapeutic targets to manage OAB syndromes. However, in contrast to the relatively well characterized actions of ATP in the urinary bladder both in physiological and pathological conditions, limited information is available on the role of its breakdown product, adenosine. First reports of the effect of adenosine in the lower urinary tract suggested that it reduces the tone and the spontaneous activity of the urinary bladder in different species (Acevedo et al., 1992; Brown et al., 1979; Burnstock et al., 1978; King et al., 1997; Nicholls et al., 1992), including humans (Rubinstein et al., 1998). Although information regarding location of adenosine receptors on the detrusor is still scant here are reports suggesting that low

affinity A_{2B} receptors are abundantly expressed in the rat bladder smooth muscle (Stehle et al., 1992; Yu et al., 2006), while our group recently showed that in the human detrusor the A_{2A} receptor seems to be the more abundant receptor subtype (Silva-Ramos et al., 2015a). Conflicting pharmacological evidence for the presence of A_1, A_{2A} and A_3 receptors in detrusor smooth muscle fibers subsist in the literature depending in the animal species and the experimental settings (Gopalakrishnan et al., 2002; Vesela et al., 2011; Yang et al., 2000). Despite this, a remarkable parallelism seems to exist between the potency of adenosine and β_3-adrenoceptor agonists to inhibit transmitter release and to promote relaxation of myogenic detrusor contraction (see above). Data from our group show that relaxation of human detrusor strips pre-contracted with ACh require 30-fold higher concentrations (milimolar range) of adenosine than the inhibitory effect of the nucleoside on evoked [^{3}H]ACh release operated by prejunctional A_1 receptors (Silva-Ramos et al., 2015a). This difference was even more significant in the bladder of patients with benign prostatic hyperplasia, a pathological condition that may accompanied by OAB-like symptoms. It, thus, appears that bladder outlet obstruction (BOO) increases the sensitivity of nerve-evoked transmitter release to exogenous adenosine receptor agonists, a situation that occurs in parallel to the up-regulation of inhibitory A_1 receptors localized in VAChT-positive cholinergic nerve fibers. It remains to be investigated whether up-regulating of the A_1 inhibitory control of ACh release also occurs in OAB patients, which might contribute to explain the clinical success of β_3-adrenoceptor agonists, including isoprenaline, was very similar in patients with low bladder compliance, hyperreflexic bladders and controls (Igawa et al., 2001), but the relaxing effect of clembuterol was significantly greater in neurogenic-contracted bladder strips from patients with urodynamically confirmed urge incontinence compared to continent patients (Hudman et al., 2001). Amplification of β_3-adrenoceptor signaling by adenosine A_1 receptors may again further relevance considering that no significant changes in the mRNA expression of β_3-adrenoceptors were observed in humans and rats with BOO (Barendrecht et al., 2009; Nomiya et al., 2003), yet β_3-adrenoceptors agonists decreased more efficiently non-voiding contractions in obstructed bladders *in vivo* (Igawa and Michel, 2013).

Using western blot analysis and immunofluorescence localization, Yu and col. Detected the expression of all four types of adenosine receptors in the bladder urothelium of female Sprague-Dawley rats (Yu et al., 2006). The A_1 receptors were

predominantly localized to the apical membrane of umbrella cells, whereas A_{2A}, A_{2B} and A_3 receptors were expressed intracellularly or on the basolateral membrane of umbrella cells and the plasma membrane of underlying cell layers. Here, we show that the A_1 receptor is abundantly expressed in the human urothelium (*see* Figure 20A). The A_1 receptor immunoreactivity was also observed in the underlying submucosal connective tissue, yet the staining density was weaker compared to the urothelial layer. This findings agrees with the work of Yu and col. showing that suburothelial tissue elements (possibly connective cells, myofibroblasts or blood vessesls) are more strongly labeled with the A_{2A} antibody (Yu et al., 2006). Based upon quantitative PCR experiments, Nomiya and Yamaguchi (2003) claimed that the β_3-adrenoceptor accounts for more than 95% of all β-adrenoceptor mRNA in the human bladder (Nomiya and Yamaguchi, 2003; reviewed in Michel and Vrydag, 2016), which is consistent with the extensive β_3 receptor protein immunoreactivity found by us in the human detrusor (Figure 20).

However, a more recent quantitative analysis under highly standardized conditions suggested comparable β_1, β_2 and β_3 adrenoceptors mRNA expression in the human bladder (Uhlén et al., 2015). Anyway, the use of selective β_3 adrenoceptor agonists, such as like mirabegron (Takasu et al., 2007); *reviewed in* Michel and Vrydag, 2006), and antagonists, like L748,337 (Candelore et al., 1999), reduces tremendously any lack of discriminative power of less selective compounds (*e.g.* isoprenaline, SR59230A) regarding β-adrenoceptor subtypes in human functional studies (Baker, 2010; Hoffamnn et al., 2004). Likewise, neither dobutamine (a β_1 receptor agonist) nor procalterol (a β_2 receptor agonist) produced significant relaxation in the human detrusor (Igawa et al., 1998), suggesting that the β_3-adrenoceptor is the main responsible for the adrenergic inhibitory tone in human bladder. Manipulation of β_2-adrenoceptors activity with fenoterol (agonist) and ICI118,551 (antagonist) was virtually devoid of effect on [^{3}H]ACh release from the stimulated rat detrusor and produced only minor changes in the urodynamic parameters evaluated in the anaesthetized rat compared to isoprenaline and CL316,243. Thus, our findings suggest that β_2-adrenoceptors contribute to a minor extend, if any, to control cholinergic neurotransmission and bladder reactivity in the rat.

Besides the presence of β_3-adrenoceptors on smooth fibers of the human detrusor (see above) these receptors are equally abundant in vimentin-positive

interstitial cells of the suburothelium; which may correspond to the immunoreactivity we have detected in the suburothelial layer (Figure 20). A minor density of expression of β_3-adrenoceptors was found in the urothelium. Data from previous studies suggest that the majority of these receptors are present in apical umbrella cells (Limberg et al., 2010; Otsuka et al., 2008); high magnification micrographs obtained by our group showed that β_3 and A_1 receptors co-localize with CK20, an apical urothelial cell marker, in the human bladder (unpublished observations). The close proximity of adenosine A_1 and β_3 receptors in umbrella cells may explain their crosstalk to decrease sensitivity in the urinary bladder to isoprenaline-containing saline distension in the anaesthetized rat, which resulted in reduction of the voiding frequency and in enhancement of the bladder storage capacity. Although isoprenaline-induced of the micturition cycle was prevented by co-application with selective β_3-and A_1 receptor antagonists SR59230A and DPCPX, respectively, one cannot exclude the participation of suburothelial structures endowed with the two receptors, including afferent and efferent nerves (or both) and interstitial cells that may influence bladder spontaneous activity and detrusor tonus (Limberg et al., 2010). In support of this possibility, our results showed that both blockers were equally effective when instilled into the bladder lumen or when induced intravenously. Moreover, intravenous application of isoprenaline also reduced the intra-vesical pressure and increased bladder storage capacity in a model of distension-induced bladder activity under isovolumetric conditions in urethane-anaesthetized rats (Lecci et al., 1998).

Interestingly, previous studies demonstrated that β_3-adrenoceptors inhibit detrusor contractions of the pig urinary via the urothelium (Masunaga et al., 2010), most probably through the release of an urothelium-derived factor of unidentified nature as it has been proposed in porcine and human bladders (Murakami et al., 2007; Otsuka et al., 2008). Our best hypothesis is in favor of adenosine, which might be released in response to urothelial β_3-adrenoceptors activation via equilibrative nucleoside transporters existing at the basolateral surface of epithelial cells (Loffler et al., 2007), but not evidence still exist to exclude other mechanisms leading to urothelial adenosine accumulation, namely the release of ATP and cyclic AMP. Differences in the kinetics of the extracellular catabolism of adenine nucleotides by ecto-nucleotidases between luminal and abluminal sides of the human urothelium indicate that adenosine biosynthesis predominates in the basal layer of the

urothelium where ecto-5'-nucleotidases/CD73 is dominantly expressed (Silva-Ramos et al., 2015b). This feature strengthens our former hypothesis that adenosine is predominantly originated via the nucleoside transport system in the more superficial layers of the human urothelium. Once in the extracellular milieu adenosine starts an inhibitory signaling cascade at more superficial urothelial layers via the activation of neighboring cells endowed with A_1 receptors. It has been demonstrated that adenosine negatively modulates stretched-induced ATP release over many minutes by modulating exocytosis in the apical membrane of umbrella cells (Yu et al., 2006). A more acute (within seconds) effect to modulate distension-induced ATP release from the urothelium has also been suggested (Dunning-Davies et al., 2013). Distension-induced ATP release was decrease by adenosine (1-10 μM) and enhanced by adenosine deaminase and DPCPX, but not by blocking A_2 receptors with 3,7-dimethyl-1-propargyl-xanthine (DMPX). The mechanism by which adenosine reduces urothelial ATP release remains unknown, but it might depend on changes in the transepithelial potential by interference with mechanosensitive Na^+ channels (identified as amiloride-sensitive epithelial Na^+ channels or ENaC), which may ultimately favor Ca^{2+} outflow via Na^+/Ca^{2+} exchange in urothelial cells (Wu et al., 2011). These findings support a dominant role of adenosine A_1 receptors in regulating ATP release from the urothelium and, thus, in the control of the sensory filling phase of the micturition cycle. In fact, prolongation of the intercontraction interval without affecting the amplitude of voiding contractions was observed when A_1 receptor agonists were applied into the lumen of the urinary bladder in the rat *in vivo* (Kitta et al., 2014).

Alternatively, the production and release of NO from urothelial cells may follow β-adrenoceptors activation leading to increases in intracellular Ca^{2+} triggered by activation of the cyclic AMP pathway (Birder et al., 2002). However, these authors did not specifically test the role of any selective β_3-adrenoceptor agonist/antagonist. Because NO has only minimal relaxing effects on bladder smooth muscle (Andersson and Persson, 1995), it was suggested that NO can down-regulate bladder activity by suppressing excitability of sensory nerve afferents (Pandita et al., 2000). Even though we cannot discount the participation of NO in the β_3-adrenoceptor-induced inhibitory pathway controlling bladder activity in the anaesthetized rat, blockade of the isoprenaline effect by DPCPX strongly suggest that adenosine A_1 receptors play a more relevant role.

Controversy, still exists in whether the prototypical activation of adenylyl cyclase and cyclic AMP generation coupled to β_3-adrenoceptors activation is the exclusive mechanism triggering the inhibitory effects on urinary bladder tone (*reviewed in* Michel and Vrydar, 2006; Yamaguchi and Chapple, 2007). Other possibilities include modulation of membrane potential, ion-channels activity and intracellular ion concentrations. Isoprenaline was found to hyperpolarize the cells, prevent action potentials and decrease Ca^{2+} transients. Activation of potassium current may cause membrane hyperpolarization and relaxation of the urinary bladder, but differences in species and in the stimulation protocol used by distinct research groups impair more positive conclusions. Although large conductance calcium-activated K^+ channels (BKCa channels) have been associated with cyclic AMP-independent relaxation induced by β_3-adrenoceptor agonists in pre-contracted rat detrusor with high KCl (Uchida et al., 2005), no evidence of such a mechanism exits in the human bladder. Notwithstanding this, data from the present study indicate that both isoprenaline (via β_3-adrenoceptors activation) and the receptor-independent adenylyl cyclase activator, forskolin, decreased evoked [^{3}H]ACh release from rat bladder strips without urothelium. Furthermore, we showed that the inhibitory effect of isoprenaline was prevented in the presence of two equilibrative nucleosides transport inhibitors, NBTI and dipyridamole. Likewise, dipyridamole also prevented that the inhibitory effect of mirabegron on evoked [^{3}H]ACh release from the human detrusor. Distribution of the immunoreactivity against NBTI-sensitive equilibrative nucleoside transporter 1 (ENT1) on the plasma membrane of detrusor smooth muscle fibers in close proximity to β_3-adrenoceptors contrast with the predominance of NBTI-insensitive (ENT2) immunostaining surrounding smooth muscle bundles. Thus, imaging and functional studies suggest that β_3-adrenoceptor-induced adenosine release from detrusor bladder strips result from translocation of the nucleoside across the plasma membrane via the ENT1 subtype. This is possible because increased cyclic AMP levels in stimulated cells are effectively degraded into AMP and adenosine by highly active phosphodiesterases and 5'-nucleotidase, respectively, to control its intracellular effects. Consequently, this raises the transmembrane gradient forcing the nucleoside outflow to the extracellular compartment. Alternatively, cyclic AMP actively transport outside cells via several members of the group of ATP-binding cassette (ABC) transporters (Chen et al., 2001; Guo et al., 2003; Jedlitschky et al., 2000; Van Aubel et al., 2002). Even though this mechanism has been

described at the urinary bladder (Uchida et al., 2005) and cyclic AMP can originate adenosine in the extracellular space in many cell types through the sequential hydrolysis by two ectoenzymes, ecto-nucleotide pyrophosphatase/ phosphodiesterase 1 (ENPP1) and ecto-5'-nucleotidase/CD73 (see *e.g.* Chiavegatti et al., 2008; Sassi et al., 2014), our results demonstrate that the activity of ENPP1 converting cyclic AMP into AMP is less productive than ecto-5'-nucleotidase/CD73 that is responsible for AMP dephosphorylation into adenosine in the detrusor of both humans and rats. Given that low adenosine amounts resulted from the extracellular cyclic AMP-adenosine pathway in human and rat detrusor strips, it might not account significantly for the extracellular adenosine accumulation detected following β_3-adrenoceptors activation with isoprenaline or mirabegron.

CONCLUSION

In conclusion, data from the present study suggest that inhibition of cholinergic neurotransmission by β_3-adrenoceptors activation results from adenosine release from stimulated detrusor smooth muscle fibers, via ENT1, leading to retrograde activation of prejunctional A_1 receptors in human and rat urinary bladder. β_3-adrenoceptor agonists may also exert a clinically useful effect by fine-tuning regulating the sensory bladder drive during urine storage through urothelium-derived adenosine release from mechanically-sensitive umbrella cells, which are also endowed with ENT1 nucleoside transporters. The molecular mechanism underlying adenosine release downstream β_3-adrenoceptor-induced adenylyl cyclase activation and cyclic AMP generation in smooth muscle fibers and urothelial cells remains to be elucidated. Notwithstanding this, we propose here a novel mechanism involving endogenous adenosine release and A_1 receptor activation which contribute to explain, at least partially, the therapeutic success of β_3-adrenoceptor agonists on OAB symptoms. β_3-adrenoceptor agonists with the participation of endogenous adenosine may exert inhibitory effects on bladder functions increasing the storage capacity and prolonging the micturition interval, without affecting the voiding pressure or post-void residual volume. These drugs may act both (a) in the sensory bladder drive operated by mechanically-sensitive urothelial cells, sensory nerve afferents and interstitial pacemaker cells, and (b) on the efferent motor component resulting in the

reduction of non-voiding cholinergic microcontractions and in the increase in bladder storage capacity by relaxing the detrusor. Apparently these processes are affectively cut-short during the voiding command by yet unknown mechanisms, which may involve the unrestrained parasympathetic boost (reviewed in Yamaguchi and Chapple, 2007); in fact, β_3-adrenoceptor agonists do not affect voiding urodynamics paramaters (maximum urinary flow and detrusor pressure at maximal urinary flow) in clinical settings (Nitti et al., 2013) and we did not observed any changes in the duration an amplitude of voiding urinary contractions in response to isoprenaline and CL316,243 in the anaesthetized rat, nor these parameters were affected by blockage of adenosine A_1 receptors with DPCPX. Thus, we propose that pharmacological manipulation of endogenous adenosine levels and/or A_1 receptor activation may act synergistically with β_3-adrenoceptors activation to control bladder overactivity.

ACKNOWLEDGEMENTS

This research was partially supported by Fundação para a Ciência e a Tecnologia (FCT, FEDER funding, projects PTDC/SAU-OSM/104369/2008, REEQ/1264/SAU/2005, Pest-OE/SAU/UI0215/2014 and UID/BIM/4308/2016), Associação Portuguesa de Urologia (APU) and University of Porto/Caixa Geral de Depósitos (Investigação Científica na Pré-Graduação). The funders had no role in study design, data collection and analysis, decision no publish, or preparation of the manuscript. Isabel Silva was in receipt of a PhD Studentship from FCT (SFRH/BD/88855/2012). The authors wish to thank Mrs. Helena Costa e Silva and Belmira Silva for their valuable technical assistance.

PAPER 4

Manuscript in preparation

Cyclic AMP activation of the exchange protein EPAC mediates β_3-adrenoceptor inhibition of cholinergic neurotransmission in human and rat urinary bladders

Isabel Silva[1,2], Maria Teresa Magalhães-Cardoso[1,2], Fátima Ferreirinha[1,2], Sílvia Moreira[1,2], Ana Filipa Costa[1,2], Miguel Silva-Ramos[1,2,3] and Paulo Correia-de-Sá[1,2]*

[1]Laboratório de Farmacologia e Neurobiologia, [2]Center for Drug Discovery and Innovative Medicines, Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto and [3]Serviço de Urologia, Centro Hospitalar do Porto, Porto, Portugal

* Corresponding author: farmacol@icbas.up.pt

Contribution of authors: PCS supervised the project, designed the experiments, and analyzed data. IS and PCS wrote the paper. IS and MTMC performed and analyzed data from nucleosides release experiments by HPLC. IS performed and analyzed data from acetylcholine and adenosine release experiments. AFC, SM and IS performed and analysed data from in vivo rat bladder cystometry experiments. IS and FF performed Western blot analysis, immunofluorescence staining and confocal microscopy observations. MSR recruited the patients and collected bladder samples. IS, AFC, SM, MTMC, FF, MSR and PCS interpreted data, discussed the clinical implications and commented on the manuscript at all stages.

ABSTRACT

Previously our group showed that activation of the β_3-adrenoceptor with therapeutic doses of mirabegron and isoprenaline downregulates cholinergic neurotransmission in human and rat bladders, respectively, by indirectly increasing adenosine release via equilibrative nucleoside transporters and, subsequent, activation of pre-synaptic inhibitory A_1 receptors. β_3-adrenoceptors, via $G_{\alpha s}$ proteins, are usually coupled to the adenylate cyclase / cyclic AMP (AC/cAMP) transduction pathway. The cellular effects of cyclic AMP (cAMP) are most often attributed to protein kinase A (PKA), but increasing evidence suggests the involvement of EPAC, an exchange protein directly activated by cAMP. Our aim in this study was to investigate the participation of these two cyclic AMP-dependent signalling pathways on β_3-adrenoceptor-induced adenosine outflow and inhibition of acetylcholine (ACh) release in human and rat urinary bladders. In human urothelium-denuded detrusor

samples, mirabegron-induced adenosine outflow was prevented by blocking β_3-adrenoceptors with L748,337 (30 nM) and the equilibrative nucleoside transporter (ENT1) with dipyridamole (0.5 µM). The same occurred by inhibiting PKA and EPAC with H-89 (10 µM) and ESI-09 (10 µM), respectively. The inhibitory effects of mirabegron and isoprenaline on evoked [^{3}H]ACh release from human and rat detrusor samples, respectively, were significantly attenuated (P<0.05) by ESI-09 (10 µM), but not by H-89 (10 µM). Direct activation of adenylyl cyclase with forskolin (FSK, 0.03-10 µM) mimicked isoprenaline-induced inhibition of [^{3}H]ACh release from the rat detrusor and the same was verified in the voiding frequency of urethane-anaesthetized rats. The inhibitory action of FSK was prevented by DPCPX (100 nM, a selective A_1 receptor antagonist), dipyridamole (0.5 µM) and ESI-09 (10 µM), but not by H-89 (10 µM), in both models. Data suggest that inhibition of cholinergic neurotransmission by β_3-adrenoceptors is downstream mediated by the exchange protein directly activated by cAMP (EPAC) both in human and rat urinary bladders. The link between EPAC activation and adenosine release via equilbrative nucleoside transporters remains to be elucidaded.

INTRODUCTION

β_3-adrenoceptor agonists, like as mirabegron, belong to a new class of drugs which proved their utility in the treatment of the overactive bladder syndrome (Chappel et al., 2014). However, the localization and mechanism of action of these receptors in the urinary bladder is still a matter of debate. The localization of β_3-adrenoceptors on bladder cholinergic nerves positive for the vesicular acetylcholine transporter (VAChT) has been recently suggested (Coelho et al., 2017). However, this finding is not corroborated by other research groups showing a preferential localization of β_3-adrenoceptors on detrusor smooth muscle fibres of the urinary bladder of humans, rats and mice, as determined by immunofluorescence confocal microscopy using multiple antibodies targeting different receptor epitopes (Igawa et al., 1998; Igawa et al., 1999; Griffin et al., 2017; Silva et al., 2017). Elucidation of the mechanism of action of the β_3-adrenoceptor agonist, mirabegron, is deemly needed, since it has been considered the first of a new class of compounds for the treatment of overactive bladder syndromes which molecular mechanism may be different from anti-muscarinic drugs (Angulo et al., 2013). The paradoxical localization of β_3-

adrenoceptor on detrusor smooth muscle fibers *vs.* cholinergic nerves (see above) and the fact that peak plasma levels of mirabegron when administrated in therapeutic (50 mg daily) doses (83-167 nM) are below its potency (EC_{50}~1-3 μM) to relax detrusor smooth muscle strips (Michel and Korstanje, 2016), raise puzzling questions about the molecular mechanism of action of mirabegron which are not yet resolved in the literature (Andersson, 2017; Okeke et al., 2017).

D'Agostino et al. (2015) showed that β_3-adrenoceptors activation reduced [^{3}H]ACh release from stimulated cholinergic bladder nerves. Recently, our group added some valuable information demonstrating that activation of β_3-adrenoceptors in urothelium-denuded detrusor samples of both humans and rats favours the release of adenosine, via equilibrative nucleoside transporters (ENT; Silva et al., 2017). These findings clearly indicate that cholinergic inhibition by β_3-adrenoceptor agonists is indirectly mediated by adenosine acting on prejunctional inhibitory A_1 receptors located on cholinergic bladder nerves (Silva-Ramos et al., 2015a; Silva et al., 2017). This was concluded because the ENT inhibitor, dipyridamole, and the selective adenosine A_1 receptor antagonist, 1,3-dipropyl-8-cyclopentyl xanthine (DPCPX), fully prevented [^{3}H]ACh release inhibition by mirabegron, when this compound was applied in the low nanomolar concentration range. It remains, however, to be determined the exact mechanism underlying adenosine release downstream β_3-adrenoceptors activation in the urinary bladder and whether it is modified under pathological conditions. Patients with bladder outflow obstruction (BOO) secondary to benign prostatic hyperplasia (BPH) often exhibit bladder overactiviity remaining after prostatectomy (reviewed in Komninos and Mitsogiannis, 2014). Our group showed that the bladder of these patients exhibit increased amounts of inhibitory A_1 receptors on cholinergic nerve terminals (Silva-Ramos et al., 2015a), which according to this theory make them good candidates for the therapeutic use of β_3-adrenoceptor agonists, like mirabegron, to control bladder overactivity (Silva et al., 2017).

Activation of β-adrenoceptors is positively coupled to adenylyl cyclase (AC), via $G_{\alpha s}$-proteins, resulting in the accumulation of intracellular cyclic AMP (cAMP) (Wallukat, 2002). Mirabegron increased intracellular cAMP accumulation in CHO cells stably transfected with rat and human β_3-adrenoceptors (Atanaka et al., 2013). In the bladder, both cAMP-dependent and cAMP-independent pathways have been reported to be involved in the relaxation of the detrusor smooth muscle (Uchida et al.,

2005). Moreover, the intracellular cAMP transduction system may be impaired in pathological conditions (e.g. hypoxia) (Jóźwiak-Bębenista et al., 2016).

The cellular effects of cAMP are most often attributed to protein kinase A (PKA) and cyclic nucleotide-regulated ion channels (Wang et al., 2017). Activated PKA phosphorylates myosin light chain kinase (MLCK), thereby impairing Ca^{2+}-calmodulin-dependent activation, which reduces myosin light chain (MLC) phosphorylation and, hence, the muscle tone (Andersson and Arner, 2004; Hashitani et al., 2004). Bladder relaxation caused by β-adrenoceptor agonists may involve PKA-mediated phosphorylation of large conductance Ca^{2+}-activated K^+ channels (BK_{Ca}; Petkov, 2014) leading to cell membrane hyperpolarization and to decreases in Ca^{2+} influx via voltage-dependent channels (Wegener et al., 2004). Despite these possibilities, detrusor relaxation owing to the AC/PKA pathway downstream activation of β-adrenoceptors has been questioned (Frazier et al., 2005), raising the hypothesis that PKA-independent effects might also be involved.

Novel cyclic AMP targets have emerged to explain cell responses that are insensitive to PKA inhibition (de Rooij et al., 1998; Kawasaki et al., 1998; reviewed in Dekkers et al. 2013). The exchange protein directly activated by cAMP (EPAC) is a cyclic AMP-regulated guanine nucleotide exchange factor (GEF) that favors GDP/GTP exchange and, thereby, activation of small Ras-like GTPases, such as Rho, Rac and Ras. Interestingly, EPAC is known to control distinct cellular responses, including calcium handling, cell proliferation, differentiation, migration, fibrogenic and inflammatory responses, which might contribute to bladder dysfunctions.

The lack of understanding about the molecular targets of β_3-adrenoceptor agonists and their putative repercussions in the treatment of lower urinary tract symptoms (LUTS) led us to investigate the participation of the two main cyclic AMP-dependent pathways on β_3-adrenoceptor-induced adenosine outflow and inhibition of electrically-evoked [^{3}H]ACh release from human and rat urinary bladder samples. To test if a similar pathway participates in the inhibition of the sensory bladder drive caused by β_3-adrenoceptors, we performed *in vivo* cystometry recordings in urethane-anaesthetized male rats. We also evaluated the effect of the β_3-adrenoceptor agonist, mirabegron, on evoked [^{3}H]ACh release from detrusor strips isolated from men with BOO due to BPH, in order to test if this mechanism is operative in patients with LUTS.

Keywords: β_3-adrenoceptors, cAMP, PKA pathway, Epac pathway, urinary bladder.

MATERIAL AND METHODS

Animals

Animal care and experimental procedures were in accordance with the guidelines prepared by Commitee for the Update of the Guide for the Care and Use of Laboratory Animals (National Research Council, USA) and followed the European Communities Council Directive (86/609/EEC). All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals. A total of twenty animals were used in the experimental described here. Male rats (Wistar, 200-300 g; Charles River, Barcelona, Spain) were kept at a constant temperature (21ºC) and a regular light (06:30-19:30 h) – dark (19:30-06:30 h) cycle, with food and water provided *al libitium*.

Human bladder samples

Samples of the human detrusor were collected from the bladder dome of five male organ donors (38±4 years of age) at the time of harvesting organs for transplantation; detrusor samples were also obtained from six consecutive patients (65±1 years of age) with bladder outlet obstruction due to BPH who underwent transvesical prostatectomy. Collected samples were immediately placed at 4-6ºC 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 $K_2HPO_4.3H_2O$, 15 mM KCL, and 10 mM $NaHCO_3$, pH 7.4) and transported to the laboratory. Experiments were performed within the first 24h after collection, which corresponds to the tissue viability window. This study and all its procedures were approved by the Ethics Committees of CHP and ICBAS-UP and were authorized by the National Transplantation Committee. Regarding decease organ donation, the legal frame work allows the “Presumed Consent” stating that residents in Portugal are consenting donors for transplantation and research unless the individual previously objected

during his or her life. The investigation conforms to the principles outline in *The Code of Ethics of the World Medical Association* (Declaration of Helsinki).

Quantification of [^{3}H]ACh release

The experiments were performed in isolated detrusor muscle strips without the mucosa of both human and rat urinary bladders. The mucosa was dissected out either by blunt dissection through cleavage at the *lamina propria* or by gently rubbing the urothelium with a cotton wool swab in human and rat bladder samples, respectively (Carneiro et al., 2014; Silva-Ramos et al., 2015a; Silva et al., 2017). Full thickness isolated detrusor muscle strips (3 mm width, 5 mm length; weighting 9.2±0.5 mg (human) and 5.9±0.2 mg (rat)) were mounted in 365 µL capacity chambers of a Brandel SF-12 automated superfusion system (Valley International Corp., Austin, TX, USA) heated at 37ºC. Then, the preparations were continuously superfused with gassed (95% O_2 and 5% CO_2) Tyrode's solution (pH 7.4) containing (mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1, NaH_2PO_4 0.4, $NaHCO_3$ 11.9, glucose 11.2 and choline 0.001. After a 30 min equilibration period, cholinergic neurons were loaded during 40 min with 1 µM [^{3}H]choline (specific activity 5 µCi nmol^{-1}) under electrical field stimulation (1 Hz frequency, 0.2 ms pulse width, 75 mA) using two platinum-made grid electrodes placed above and below the muscle strip (transmural EFS stimulation). Following loading, the washout superfusion (1 ml/min) of the preparations was performed during 120 min with Tyrode's solution supplemented with the choline uptake inhibitor, hemicholinium-3 (10 µM). Tritium outflow was evaluated by liquid scintillation spectrometry (TriCarb2900TR, Perkin Elmer, and Boston, USA; % counting efficiency: 56±2%) after appropriate background substraction, using 1 ml bath samples automatically collected every 1 min using the SF-12 suprafusion system. [^{3}H]ACh release was evoked by two periods of EFS (S_1 and S_2), each consisting of 200 square wave pulses of 0.2 ms duration delivered at 10-Hz frequency. Test drugs were added 6 min before S_2 and were present up to the end of the experiments. The evoked [^{3}H]ACh release was calculated by subtracting the basal tritium outflow from the total tritium outflow during the stimulation period (see e.g. ref. Carneiro et al., 2014 and Silva et al., 2017). In control situations, S_2/S_1 ratio were 0.99±0.04 (n=6) and 0.96±0.02 (n=4) in human and rat detrusor samples, respectively.

Measurement of adenosine release

The procedures used to measure the release of adenine nucleosides (adenosine and inosine) were previously described (see e.g. Correia-de-Sá et al., 2006; Vieira et al., 2014; Silva et al., 2017). Experiments were performed in isolated rat and human detrusor strips without the mucosa using an automated perfusion system for sample collecting for given time periods, therefore improving the efficacy of HPLC (with diode array detection). After a 30-min equilibration period, the preparations were incubated with 2.8 mL gassed Tyrode's solution, which was automatically changed every 15 min by emptying and refilling the organ bath with the solution in use. In these experiments, samples retained for analysis were collected 15, 30 and 45 min after starting the experimental protocol (zero time). Isoprenaline (1 µM) and mirabegron (0.1 µM) were added to the incubation solution after the first collection (Basal, 15 min) and were kept in contact with the preparation at least for 15 min (Drug 30 min) before starting electrical field stimulation (Drug + EFS, 45 min), which consisted in 3000 square wave pulses of 1 ms duration delivered at a 10-Hz frequency. Bath aliquots (50-250 µl) were frozen in liquid nitrogen immediately after collection, stored at -20ºC (the enzymes are stable for at least 4 weeks) and analyzed within 1 week of collection by HPLC with diode array detection (Finnigan Thermo Fisher Scientific System LC/DAD, equipped with an Accela Pump coupled to an Accela Autosample, a diode array detector and an Accela PDA running the X-Calibur software chromatograph manager). Chromatographic separation was carried out through a Hypersil GOLD C15 column (5 µM, 2.1 mm x 150 mm) equipped with a guard column (5 µm, 2.1 mm x 1mm) using an elution gradient composed of ammonium acetate (5 mM, with a pH of 6 adjusted with acetic acid) and methanol. During the procedure the flow rate was set at 200 µl per min and the column temperature was maintained at 20ºC. The autosampler was set at 4ºC and 50 µl of standard or sample was injected, in duplicate, for each HPLC analysis. In order to obtain chromatograms and quantitative analysis with maximal sensibility, the diode array detection wavelength was set at 259 nm for adenosine and 248 nm for inosine.

Western blot analysis

Human and rat detrusor strips without the mucosa were homogenized in radio-immunoprecipitation assay buffer (Tris-HCl 25 mM (pH 7.6), NaCl 150 mM, sodium deoxycholate 1%, Triton-X-100 1%, sodium dodecyl sulfate (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, USA). 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 70ºC for 10 min, subjected to electrophoresis in 10% SDS-polyacrylamide gels and electrotransferred onto polyvinylidene difluoride (PVDF) membranes (MilliPore, MA, USA). Protein loads were 75 and 150 µg. The membranes were, then, blocked in Tris-buffered saline (in mM: Tris-HCl 10 (pH 7.6), NaCl 150) containing Tween 20 0.05% and bovine serum albumin (BSA) 5% and, subsequently, incubated with (Table 10) anti-A_1 receptor from Abcam (Ab75177), anti-β_3 receptor from MBL International (MC-4198) and anti-ENT1 from Alomone (ANT051) (host species: rabbit (rb)) in the above blocking buffer overnight at 4ºC. Membranes were washed three times for 10 min in TBS/Tween-20 an incubated with horseradish peroxidase-conjugated secondary antibody (1:25000; Abcam, Cambridge, UK) for 1 h at room temperature. For normalization purpose, membranes were incubated with the mouse anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12) primary antibody from Santa Cruz (1:200, SC-32233, Dallas TX, USA) following the procedures described above. Membranes were washed three times for 10 min and the antigen-antibody complexes were visualized with the Immun-Star WesternC Chemiluminescence Kit using the ChemiDoc MP imaging system (Bio-Rad Laboartories, Hercules, CA, USA).

Table 10. Primary and secondary antibodies used in the Western blot experiments.

Antigen	Code	Host	Dilution	Supplier
Primary antibodies:				
Anti-A_1 receptor	Ab75177	Rabbit	1:250	Abcam
Anti-β_3 receptor	MC-4198	Rabbit	1:50	MBL International
Anti-ENT1	ANT-051	Rabbit	1:50	Alomone
Anti-GAPDH	Sc-32233	Mouse	1:200	Santa Cruz
Secondary antibodies:				
IgG HRP anti-rb	Ab98503	Donkey	1:70000	Abcam

In vivo cystometry recordings

The experiments were carried out in urethane-anaesthetized rats (1.0-1.2 g/kg), spontaneously breathing. Core body temperature was kept between 36ºC and 38ºC with the help of a heating pad controlled by a thermosensor connected to a

rectal probe. A catheter connected to an injection pump was inserted into the left jugular vein to permit saline infusion (4 ml/h/kg) and intravenous drugs application. After exposing the urinary bladder through a median abdominal incision, a three-barrel catheter was inserted through its dome. One barrel, was connected to an automate perfusion pump for saline and/or drugs infusion; a second barrel, was attached to a pressure transducer for continuous monitoring of intravesical pressure; the third barrel was used either to drain or to close the bladder circuit in order to initiate the micturition reflex. The bladder pressure was continuously monitored on a computer screen with a PowerLab data acquisition system (Chart 5, version 4.2 software; AD Instruments, USA), which was also used to record hemodynamic and respiratory parameters in the anaesthetized rat. After surgical preparation, a 60-min equilibration period was undertaken during which saline was infused into the urinary bladder at 0.04 ml/min and allowed to freely drain out of the bladder (open circuit). The micturition reflex was initiated by closing the draining barrel while keeping intravesical infusion of saline at a constant flow rate (0.04 ml/min), which is within the range used in previous studies to obtained stable micturition cycles during continuous cystometrograms in anaesthetized rats (Carneiro et al., 2014; Honda et al., 2012; Timóteo et al., 2014; Silva et al., 2017). The flow rate was 2 to 4-fold higher than the normal urinary debit in experimental rats (15-30 ml/d) and compare to the conditions used in standard filling cystometry (urodynamic test) in humans. Voiding contractions were assumed as large-amplitude rhythmic bladder contractions accompanied by urine draining through the urethra when bladder pressure reached a certain threshold. The intercontraction interval (ICI, min) and the pressure threshold (PTh, cm of H_2O) that is require to initiate the voiding reflex are normally associated with the sensitive component of the micturition reflex (filling phase); conversely, the amplitude (A, cm of H_2O) and the duration (Δt, sec) of the voiding contractions are mostly associated with the motor component of the micturition reflex (empyting phase). For the sake of clarity, the results presented in this study will consider the percent variation of ICI values as compared to the control situation achieved after six consecutive voiding contractions of similar amplitude. Test drugs were applied either into the bladder lumen (by changing the syringe connected to the automate perfusion pump, 0.04 ml/min) or intravenously through the catheter inserted into the left jugular vein.

Drugs and Solutions

Hemicholinium-3, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), 1,9-dideoxyforskolin (1,9-ddFSK), choline chloride, 7β-acetoxy-8,13,epoxy-1α,6β,9α-trihydroxylabd-14-en-11-one (Forskolin, FSK), S-(p-nitrobenzyl)-6-thioinosine (NBTI), α-[2-(3-Chlorophenyl)hydrazinylidene]-5-(1,1-dimethylethyl)-b-oxo-3-isoxazolepropanenitrile (ESI-09) were obtained from Sigma (St Louis, MO, USA); isoproterenol hydrochloride (Isoprenaline), N-[2-[[3-(4-Bromophenyl)-2-propenyl]amino]ethyl]-5-isoquinoline sulfonamide dihydrochloride (H-89) were obtained from Tocris Bioscience (Bristol, UK); N-[[3-[(2S)-2-Hydroxy-3-[[2-[4-[(phenylsulfonyl)amino]phenyl]ethyl]amino] propoxy]phenyl]methyl]-acetamide (L-749,337) was obtained from Santa Cruz (Dallas, USA); mirabegron was obtained from Selleckchem.com (Houston, USA); dipyridamole was obtained from Boehringer Ingelheim (Germany); [methyl-^{3}H] choline chloride (ethanol solution, 80.6 Ci nmol^{-1}) was obtained from PerkinElmer (Boston, USA). DPCPX was dissolved in a 5 mM stock solution in 99% dimethylsulfoxide (DMSO) + 1% NaOH 1 M (v/v). Mirabegron, FSK, 1,9-ddFSK, ESI-09 and NBTI were dissolved in DMSO, respectively. Other drugs were prepared in Tyrode's solution. All stock solutions were stored as frozen aliquots at -20ºC. Dilutions of these stock solutions were made daily and appropriate solvent controls were done. No statistical 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±SD, 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 was carried out using Graph Pad Prism 6.04 for Windows software (La Jolla, USA). Paired and unpaired Student's t-test with Welch's correction was used for statistical analysis when parametric data was considered. One-way analysis of variance (ANOVA) followed by the Dunnett´s *post test* was used for multiple comparisons. $P<0.05$ (two-tailed) values were considered statistically significant.

RESULTS

β3-Adrenoceptors activation favours adenosine release from human and rat urinary bladders through downstream activation of cyclic AMP-dependent pathways

Mirabegron (0.1 μM) and isoprenaline (1 μM) stimulate adenosine (plus inosine) outflow from urothelium-denuded human and rat detrusor samples, respectively (Silva et al., 2017; see Paper 3 this thesis). Like our previous results obtained in the rat detrusor, involvement of β_3-adrenoceptors leading to increases in the transport of the nucleoside through ENT was confirmed by attenuation of the mirabegron (0.1 μM)-induced adenosine outflow from the human detrusor upon blocking selectively β_3-adrenoceptors with L748,337 (30 nM, Figure 26Aii) and the ENT transport system with dipyridamole (Dipy 0.5 μM, Figure 26Aiii).

In order to test the involvement of cAMP-dependent pathways downstream activation of β_3-adrenoceptors activation, we used H-89 (10 μM) and ESI-09 (10 μM) to selectively inhibit the activation of PKA and EPAC, respectively. Preincubation with H-89 (10 μM) and ESI-09 (10 μM) prevented mirabegron (0.1 μM)- and isoprenaline (1 μM)-induced increase of adenosine (plus inosine) release from urothelium-denuded human (Figure 26A) and rat (Figure 26B) detrusor samples, respectively. These results suggest that β_3-adrenoceptors activation favours adenosine release from human and rat urinary bladders through downstream activation of PKA and EPAC.

The ability of the preparations to release adenosine (plus inosine) after preincubation with inhibitors of the adenosine transport and cAMP activated enzymes was confirmed by testing the amount of adenosine released to the incubation bath following electrical stimulation of the preparations (EFS) with 3000 pulses delivered at 10 Hz during 5 min. In the absence of test drugs, EFS increased (P<0.05) ADO+INO levels in the incubation solution from 26.0±2.4 to 47.0±5.7 $pmol mg^{-1}$ (n=6) of dry weight of preparation in the human detrusor and from 22.8±3.6 to 33.4±4.2 $pmol mg^{-1}$ (n=6) of dry weight of preparation in the rat detrusor. EFS-induced adenosine (plus inosine) outflow in the presence of test inhibitors applied at least for 15 min was not significantly (P>0.05) different from control values (data not shown).

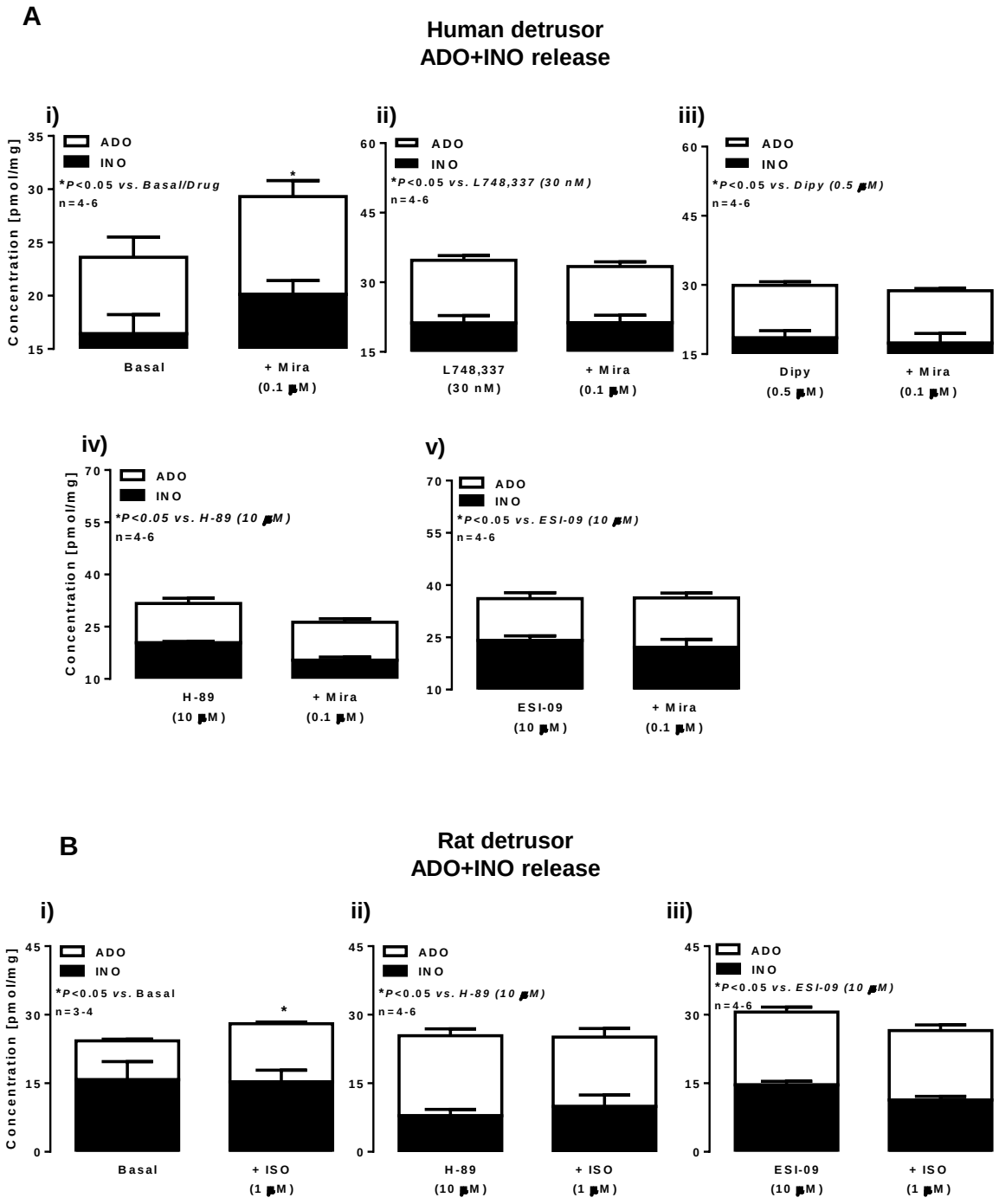


Figure 26. Effects of mirabegron (0.1 µM, **A**) and isoprenaline (Isop, 1 µM, **B**) on adenosine (and inosine) outflow from urothelium-denuned human and rat detrusor strips, respectively, in the absence and in the presence of inhibitors of PKA (H-89, 10 µM) and EPAC (ESI-09, 10 µM). The blocking effects of the β_3-adrenoceptor antagonist, L748,337 (30 nM), and the ENT1 transport inhibitor, dipyridamole (Dipy 0.5 µM), on mirabegron-induced adenosine release from the human detrusor is also shown. Mirabegron and isoprenaline contacted with the preparations for 15 min before sample collection. The inhibitors were present throughtout the assay and were applied 15 min before mirabegron or isoprenaline. The ordinates represent the concentration of adenosine (ADO, white bars)

and inosine (INO, black bars) detected by HPLC with diode array detection in samples collected from the incubation media at 15 min intervals (for details, see Materials and Methods). The data are means ± SD of four to six individuals; duplicates were performed for each individual experiment. *P<0.05 (one-way ANOVA followed by the Dunnett's post test) represent significant differences when compared to the control situation (basal/inhibitor alone).

β3-Adrenoceptors decrease electrically-evoked ACh release through a mechanism depending on EPAC activation in human and rat urinary bladders

Activation of β_3-adrenoceptors with mirabegron (0.1 μM, Figure 27A) and isoprenaline (1 μM, Figure 27B) significantly reduced (P<0.001) electrically-evoked [^{3}H]ACh release from urothelium-denuded detrusor strips isolated from humans (0.44±0.09, n=5, Figure 27A) and rats (0.49±0.06, n=4, Figure 27B), respectively (Silva et al., 2017; see Paper 3 this thesis). Inhibition of the EPAC pathway with ESI-09 (10 μM) attenuated (P<0.01) mirabegron (0.1 μM)- and isoprenaline (1 μM)-induced inhibition of evoked [^{3}H]ACh release from isolated human (0.94±0.11, n=5, Figure 27A) and rat (0.75±0.05, n=6, Figure 27B) detrusor strips, respectively. This contrasts with the lack of effect of the PKA inhibitor, H-89 (10 μM), under the same experimental conditions (Figure 27A and 27B).

Likewise, ESI-09 (10 μM, 0.85±0.07, n=6; P<0.01), but not H-89 (10 μM, 0.59±0.02, n=6; P>0.05), significantly decreased the inhibitory effect of forskolin (FSK 3 μM, 0.39±0.04, n=4; P<0.001), a direct activator of the catalytic subunit of adenylyl cyclase that mimicked the inhibitory effect of the β_3-adrenoceptor activation by isoprenaline (1 μM), on evoked [^{3}H]ACh release from isolated rat detrusor strips (Figure 27C). These results suggest that β_3-adrenoceptors-induced inhibition of evoked [^{3}H]ACh release from human and rat detrusor samples depends on downstream EPAC activation.

To confirm the specificity of the inhibitory effect of FSK (3 μM) on cAMP-dependent pathways, we tested the effect of 1,9-dideoxyforskolin (1,9-ddFSK, 3 μM), a FSK analogue that is inactive on adenylyl cyclase (Laurenza et al., 1999). Figure 27C shows that 1,9-ddFSK (3 μM) did not significantly inhibit the electrically-evoked [^{3}H]ACh release from the isolated rat detrusor (0.73±0.06, n=5; P>0.05) under the same experimental conditions. The absence of effect of 1,9-ddFSK on evoked [^{3}H]ACh release was also observed in longitudinal muscle-myenteric plexus preparations of the rat ileum (Duarte-Araújo et al., 2004).

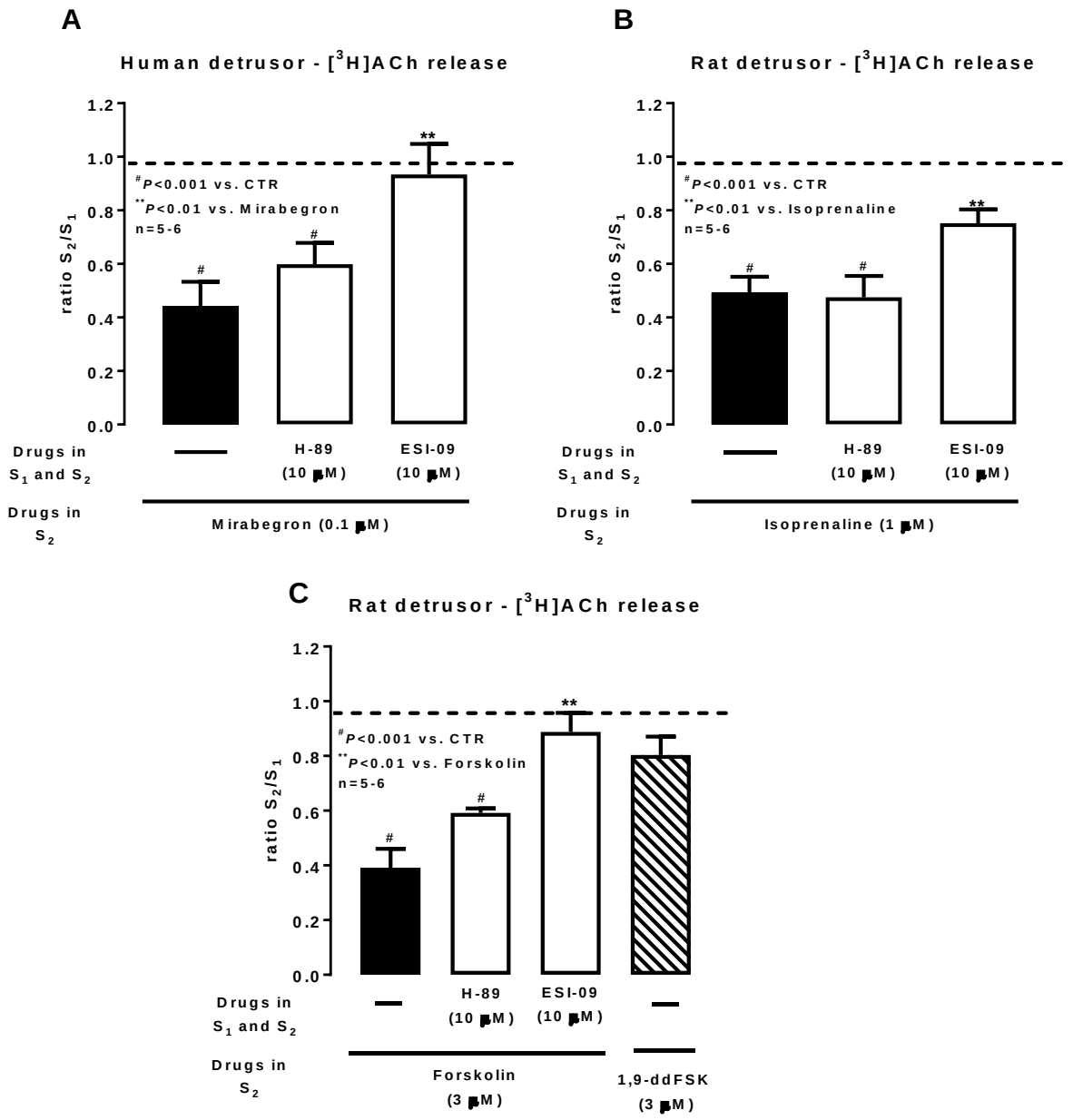


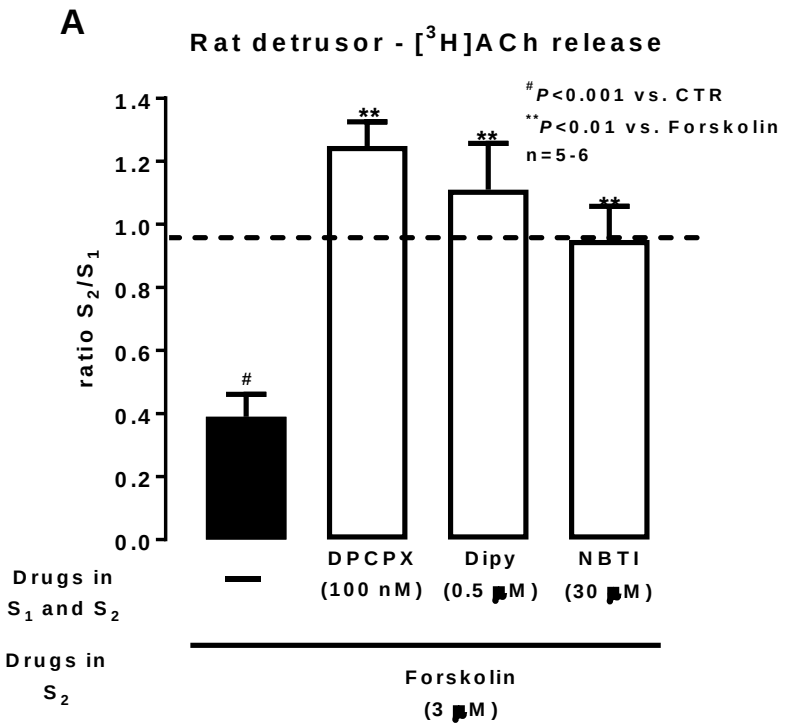
Figure 27. **(A)** Changes in the inhibitory effects of mirabegron (0.1 μM), **(B)** isoprenaline (1 μM), and **(C)** forskolin (3 μM) on electrically-evoked [^{3}H]ACh release from urothelium-denuded human and rat detrusor strips, respectively. [^{3}H]ACh release was elicited by electrical field stimulation (10 Hz, 200 pulses of 0.2 ms duration) twice, starting at 4th (S_1) and 13th (S_2) minutes after the end of washout (zero time). Mirabegron (0.1 μM), isoprenaline (1 μM), and forskolin (3 μM) were applied 6 min before S_2 either in the absence or in the presence H-89 (10 μM, a PKA inhibitor) or ESI-09 (10 μM, an EPAC inhibitor) throughout the assay, including S_1 and S_2. For comparison purposes, we also tested the effect of the FSK inactive analogue on adenylyl cyclase, 1,9-dideoxyFSK (1,9-ddFSK, 3 μM), on [^{3}H]ACh release from rat detrusor strips. Ordinates are changes in S_2/S_1 ratios compared to the S_2/S_1 ratio obtained without addition of any drug (dotted horizontal line). The data are means±SD of an *n* number of individuals. $^{\#}P$<0.001 (one-way ANOVA followed by the Dunnett's post test) represent significant differences when compared to the control situation; $^{**}P$<0.01 (one-way ANOVA followed by

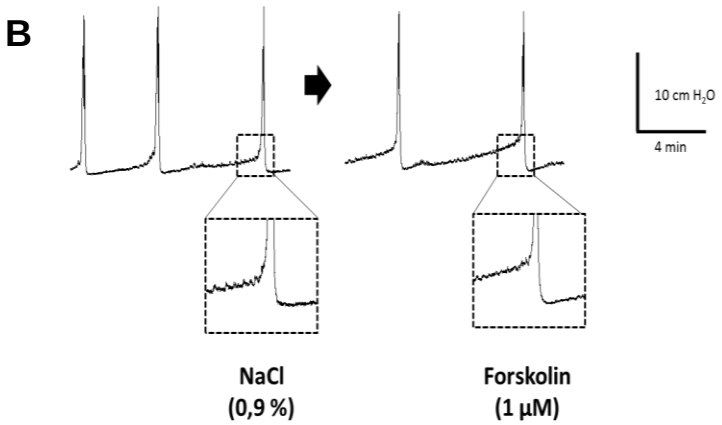
the Dunnett's post test) represent significant differences when compared to the inhibitory effect of mirabegron, isoprenaline or FSK, respectively.

Direct activation of adenylyl cyclase with FSK (3 μM) also decreases cholinergic neurotransmission depending on adenosine outflow via equilibrative nucleoside transporters and stimulation of A_1 receptors

Data from our previous study demonstrate that inhibition of [^{3}H]ACh release by β_3-adrenoceptors activation is indirectly mediated by adenosine outflow via equilibrative nucleoside transporters (ENT) from stimulated detrusor smooth muscle fibers and retrograde activation of prejunctional A_1 receptors in both human and rat urinary bladders (Silva *et al.*, 2017). Here, we show that direct activation of adenylyl cyclase with FSK (3 μM) mimicked the inhibitory effect of isoprenaline (1 μM) on evoked [^{3}H]ACh release from urothelium-denuded rat detrusor strips (Figure 27A). Like that occurring with isoprenaline (Silva *et al.*, 2017), the inhibitory effect of FSK (3 μM, 0.39±0.04, n=4; P<0.001) on evoked [^{3}H]ACh from the rat detrusor was reversed upon blocking prejunctional prejunctional A_1 receptors with 1,3-dipropyl-8-cyclopentylxanthine (DPCPX, 100 nM; 1.25±0.08, n=7; P<0.01) (Figure 28A). This result suggests that FSK-induced decrease of evoked [^{3}H]ACh release in the rat detrusor also depends on the activation of inhibitory adenosine A_1 receptors.

In many tissues, including the rat bladder, intracellular cAMP is rapidly degraded by phosphodiesterases into AMP and adenosine inside cells, which translates into increased amounts of adenosine released to the extracellular milieu via ENT (Silva et al., 2017). Synaptic accumulation of adenosine negatively modulates ACh release from cholinergic bladder nerves (Silva-Ramos et al., 2015a). In view of this, we tested the effects of two ENT inhibitors, dipyridamole (Dipy, 0.5 μM) and S-(4-nitrobenzyl)-6-thioinosine (NBTI, 30 μM), on evoked transmitter release inhibition by FSK (3 μM) in the rat detrusor; in contrast to dipyridamole, NBTI is a selective ENT1 inhibitor with no affinity for phosphodiesterase enzymes. Figure 28A shows that the inhibitory effect of FSK (3 μM, 0.39±0.07, n=4) on evoked [^{3}H]ACh release from rat detrusor strips was reversed in the presence of dipyridamole (0.5 μM, 1.11±0.15, n=5) or NBTI (30 μM, 0.95±0.11, n=7). A similar result was obtained when isoprenaline (1 μM) was used instead of FSK (3 μM) (Silva et al., 2017; see Paper 3 this thesis).


A
Rat detrusor - [3H]ACh release
#P<0.001 vs. CTR
**P<0.01 vs. Forskolin
n=5-6
ratio S2/S1
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
#
**
**
**
DPCPX (100 nM)
Dipy (0.5 µM)
NBTI (30 µM)
Drugs in S1 and S2
Drugs in S2
Forskolin (3 µM)


B
10 cm H2O
4 min
NaCl (0,9 %)
Forskolin (1 µM)

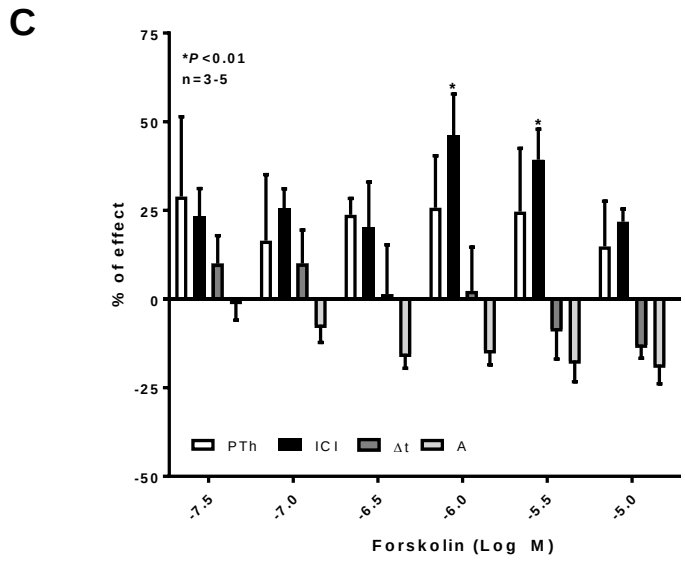

C
*P<0.01
n=3-5
% of effect
75
50
25
0
-25
-50
PTh
ICI
Δt
A
-7.5
-7.0
-6.5
-6.0
-5.5
-5.0
Forskolin (Log M)

Figure 28. **(A)** Changes in the effect of forskolin (FSK, 3 μM) on electrically-evoked [^{3}H]ACh release from urothelium-denuded rat detrusor strips in the absence and in the presence of the selective adenosine A_1 receptor antagonist, DPCPX (100 nM), and two equilibrative nucleoside transport inhibitors, dipyridamole (Dipy, 0.5 μM) and NBTI (30 μM). [^{3}H]ACh release was elicited by electrical field stimulation (10 Hz, 200 pulses of 0.2 ms duration) twice, starting at 4th (S_1) and 13th (S_2) minutes after the end of washout (zero time). FSK (3 μM) was added to the incubation media 6 min before S_2; DPCPX, dipyridamole and NBTI were present throughout the assay, including S_1 and S_2. Ordinates are changes in S_2/S_1 ratios compared to the S_2/S_1 ratio obtained without addition of any drug (dotted horizontal line). The data are means ± SD of an *n* number of animals. $^{\#}P<0.001$ (one-way ANOVA followed by the Dunnett's post test) represent significant differences when compared to the control situation; $^{**}P<0.01$ (one-way ANOVA followed by the Dunnett's post test) represent significant differences when compared to the inhibitory effect of FSK. **(B)** Bladder cystometry recordings during normal saline (0.9% w/v of NaCl) and forskolin (1 μM) infusion into the urinary bladder of urethane-anaesthetized rats. Large-amplitude bladder contractions correspond to voiding contractions. Traces obtained during the filling phase of the micturition reflex (dotted rectangles) were enlarged to show non-voiding spontaneous microcontractions. **(C)** Shown is the effect of increasing the concentration of forskolin (0.03-10 μM) inside the lumen of the urinary bladder on the cystometry parameters recorded: Pressure threshold (PTh), intercontraction interval (ICI), contraction duration (∆t) and amplitude (A). Control values correspond to zero percent variation. The vertical bars represent ± SD of three to five animals. $^{*}P<0.01$ (two-way ANOVA followed by the Dunnett's post test) represents significant differences as compared to the control situation (zero percent).

Instillation of FSK (0.03-10 μM) into the bladder lumen of anaesthetized rats concentration-dependently decreased the number and amplitude of non-voiding spontaneous microcontractions and the micturition frequency (Figure 28B); the latter was evidenced by prolongation of the intercontraction interval (ICI) without significantly ($P>0.05$) modifying the pressure threshold (PTh), the amplitude (A) and the duration (Δt) of voiding bladder contractions (Figure 28C). These results are similar to the cystometry recordings obtained when isoprenaline was used instead of FSK (see Silva et al., 2017). Instillation of forskolin (0.03-10 μM) into the bladder lumen did not ($P>0.05$) affect the heart rate (464±70 bpm, n=5) of urethane-anaesthetized rats compared to the saline infusion (445±93 bpm, n=5) under the same experimental conditions.

Isoprenaline-induced decrease in the voiding frequency in urethane-anaesthetized rats depends on EPAC activation

Figure 29 shows that pretreatment with the selective EPAC inhibitor, ESI-09 (10 μM), prevented the decrease in the voiding frequency (prolongation of the ICI interval) caused by instillation of isoprenaline (0.1-1000 nM) into the bladder lumen, whereas inhibition of PKA with H-89 (10 μM) had no effect. Neither of these drugs affected the urodynamic parameters when applied alone (data not shown). Instillation of H-89 (10 μM) and ESI-09 (10 μM) into the bladder lumen also did not significantly (*P*>0.05) affect the heart rate (429±28 bpm, n=4 and 416±62 bpm, n=5, respectively) of urethane-anaesthetized rats when compared to the saline infusion (425±28 bpm, n=4 and 392±69 bpm, n=5, respectively). The results suggest that the inhibitory effect of isoprenaline (0.1-1000 nM) on the distension-induced micturition reflex in the rat *in vivo* depends on EPAC, but not PKA, activation.

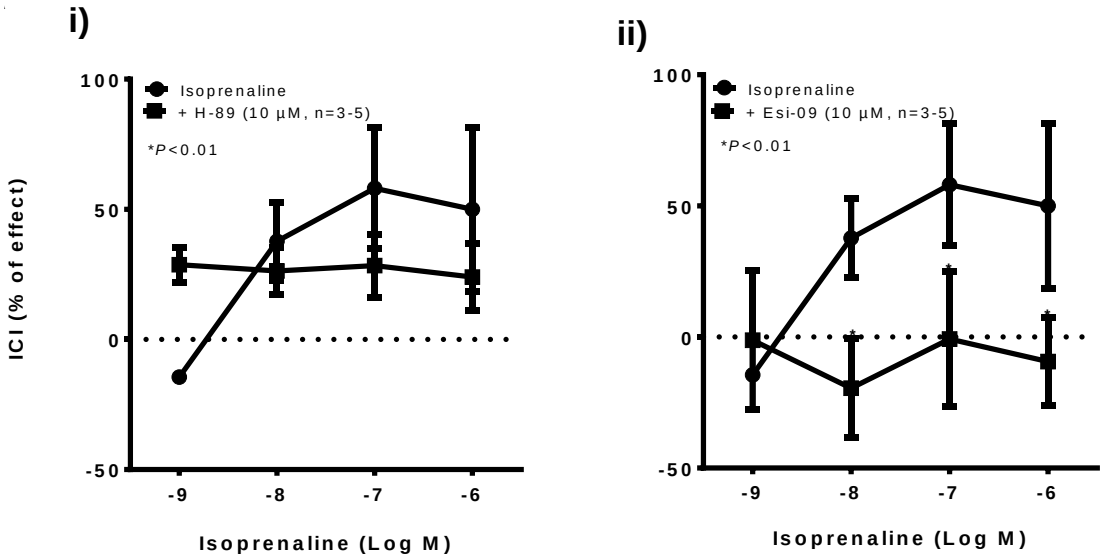


Figure 29. Shown are concentration-response curves of isoprenaline (0.01-1 μM)-induced prolongation of the intercontraction interval (ICI) of voiding contractions in the absence and in presence of (i) H-89 (10 μM) and (ii) ESI-09 (10 μM). Control values correspond to zero percent variation. The vertical bars represent SD of an *n* number of animals (shown in parenthesis). **P*<0.01 (one-way ANOVA followed by the Dunnett's post test) represents significant differences as compared to the effect of isoprenaline applied alone.

β3-Adrenoceptors activation inhibits cholinergic neurotransmission with a similar potency in control individuals and in patients with bladder obstruction due to benign prostatic hyperplasia (BPH)

In a previous study, our group showed that electrically-evoked [^{3}H]ACh release increases by 1.5-fold (*P*<0.001) in the detrusor of obstructed patients due to BPH compared to control organ donors (Silva-Ramos et al., 2015a). We also showed that the A_1 receptor is upregulated in VAChT-positive cholinergic nerve terminals of the detrusor of obstructed BPH patients, which may contribute to explain the increased inhibitory sensitivity to adenosine of nerve-evoked [^{3}H]ACh release in these patients compared to control individuals (Silva-Ramos et al., 2015a). These findings, together with the results suggesting that inhibition of evoked [^{3}H]ACh release by β_3-adrenoceptors activation is indirectly mediated by adenosine released via ENT1 and stimulation of prejunctional inhibitory A_1 receptors (Silva et al., 2017), prompted us to investigate the effect of mirabegron on evoked [^{3}H]ACh release from detrusor strips of obstructed BPH patients in order to prove that this drug has a similar mechanism of action in pathological conditions.

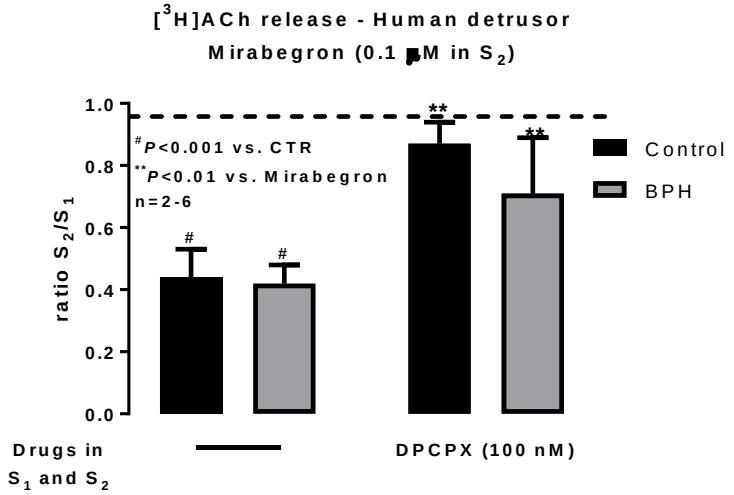


Figure 30. Inhibitory effect of selective β_3-adrenoceptors agonist, mirabegron (0.1 µM) on electrically evoked [^{3}H]ACh release from mucosal-denuded detrusor strips of organ donors (Control) and patients with benign prostatic hyperplasia (BPH); Mirabegron (0.1 µM) was applied 6 min before S_2; selective A_1 receptors antagonist, DPCPX (100 nM) was present throughout all the assay, including S_1 and S_2; [^{3}H]ACh release was elicited by electrical field stimulation (EFS, 10 Hz, 200 pulses of 0.2 ms duration) twice, starting at 4th (S_1) and 13th (S_2) minutes after the end of washout (zero time). Ordinates are change in S_2/S_1 ratios compared to the S_2/S_1 ratio obtained without of any drug (dotted horizontal line). The data are means ± SD of a *n* number of individuals; duplicates were performed for each individual experiment. $^{\#}P$<0.001 (one-way ANOVA followed by the Dunnett's post

test) represents significant diferences when compared to the control situation; **P<0.01 (one-way ANOVA followed by the Dunnett's post test) represents significant diferences when compared to the inhibitory effect of mirabegron (0.1 μM).

Figure 30 shows that the inhibitory effect of mirabegron (0.1 μM) on evoked [^{3}H]ACh release from urothelium-denuded detrusor strips had a similar magnitude in control individuals (0.44±0.09, n=5) and in obstructed BPH patients (0.42±0.06, n=5). Like that observed in control individuals (Silva et al., 2017; see also Paper 3), mirabegron (0.1 μM)-induced decrease of electrically-evoked transmitter release was prevented (0.80±0.30, n=5) by the selective β_3-adrenoceptor antagonist, L748,337 (30 nM). The inhibitory effect of mirabegron (0.1 μM) was also dependent on activation of adenosine A_1 receptors, because its effect was significantly attenuated (P<0.05) in the presence of the selective A_1 receptor antagonist, DPCPX (100 nM), both in control individuals (0.87±0.07, n=5) and obstructed BPH patients (0.71±0.18, n=2) (Figure 30).

β_3-Adrenoceptors and adenosine A_1 receptors are overexpressed while ENT1 is downregulated in the detrusor of obstructed BPH patients

Given that β_3-adrenoceptors activation by mirabegron caused a similar inhibitory effect on [^{3}H]ACh release from detrusor strips of control individuals and obstructed BPH patients, independently of the fact that adenosine A_1 receptors are overexpressed in cholinergic nerves of BPH patients, we tested the possibility that β_3-adrenoceptors and/or ENT transporters were downregulated in the detrusor of BPH patients.

The immunoblots depicted in Figure 31 show that β_3-adrenoceptors are 1.5 fold (n=4) enriched (P>0.05) in detrusor lysates of obstructed BPH patients compared to control organ donors. The MC-4198 antibody detected a major band with an apparent size of 56-59 kDa that is compatible with a glycosylated form of the human β_3-adrenoceptor. Consistent with increases in function and immunostaining detected by confocal microscopy (Silva-Ramos et al., 2015a), we show here that the A_1 receptor protein is 4.5 fold (n=4) more abundant (P<0.01) in detrusor lysates of obstructed BPH patients than in control individuals; the detected band of 45 kDa is closer to the predicted band size of 36 kDa.

On the contrary, we observed a decrease in the ENT1 protein content in detrusor lysates of obstructed BPH patients, which exhibit only 0.35 (n=3) of the total amount detected in control individuals; the detected band of 57 kDa was similar to the predicted band size of 50-55 kDa.

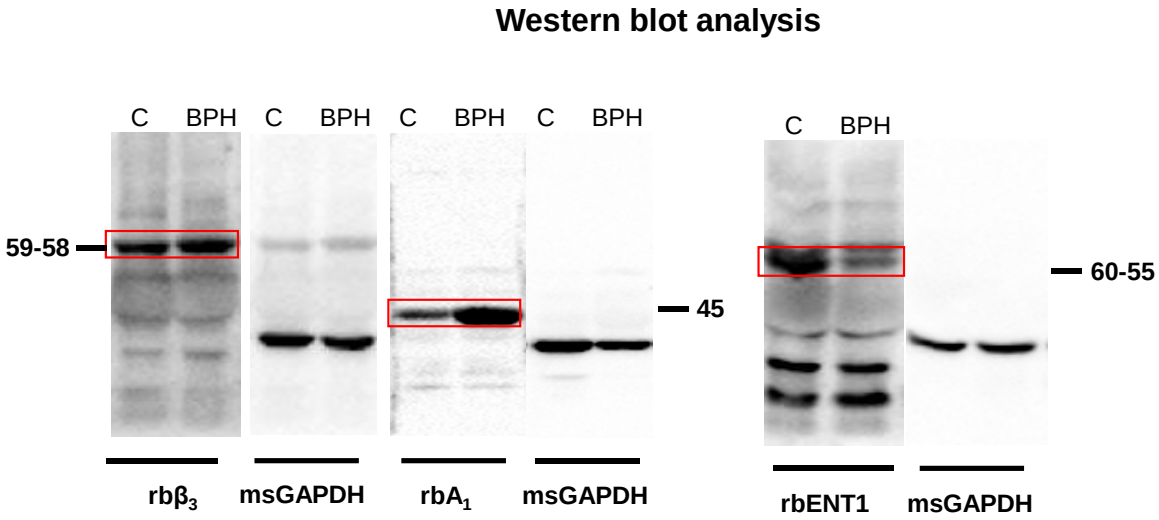


Figure 31. Shown are representative immunoblots of the β_3-adrenoceptor, the A_1-receptor and the equilibrative nucleoside transporter ENT1 in detrusor homogenates of control individuals (C) and obstructed patients due to benign prostatic hyperplasia (BPH), run in parallel. Please note that human detrusor homogenates express a prominent bands at 49,9-59,6 kDa to β_3-adrenoceptor, 45 kDa to receptor of adenosine A_1, and 55-60 kDa to ENT1 transporter. The highly conserved GAPDH enzyme (EC 1.2.1.12, MW~37kDa) was used a reference protein. Gels were loaded with 150 or 100 µg of protein. Host species for antibody production were rabbit (rb) and mouse (ms).

DISCUSSION

We show here for the first time that adenylyl cyclase (AC) activation, either directly by FSK or through stimulation of $G_{\alpha s}$-protein-coupled β_3-adrenoceptors with mirabegron and isoprenaline, promotes adenosine outflow into the extracellular milieu via ENT1 transport system through preferential downstream stimulation of the exchange protein directly activated by cAMP (EPAC) in human and rat detrusor samples. Data from this study also strengthens the theory that the inhibitory role of β_3-adrenoceptor agonists on cholinergic neurotransmission in the urinary bladder is indirectly mediated by adenosine release to the synaptic cleft leading to retrograde activation of inhibitory A_1 receptors located on cholinergic nerve terminals (Silva et al., 2017).

Selective β_3-adrenoceptor agonists, like as mirabegron or solabegron, were recently proposed as efficacious and more tolerable alternatives to anti-muscarinic drugs for the treatment of overactive bladder (OAB) syndromes (Thiagamoorthy et al., 2015). The criteria to introduce β_3-adrenoceptor agonists into the clinical setting overlooked the dispute on the molecular mechanism of action of these drugs and the precise cellular location of β_3-adrenoceptors in the human bladder. β_3-Adrenoceptors were initially found to be predominantly located on detrusor smooth muscle membranes (Fujimura et al., 1999; Igawa et al., 1998; Igawa et al., 1999). This localization was confirmed later on by immunofluorescence confocal microscopy using multiple antibodies directed towards different epitopes of the β_3-adrenoceptor both in human (Silva et al., 2017) and murine (Griffin et al., 2017). The presence of these receptors on cholinergic nerve terminals of the human bladder has recently been proposed (Coelho et al., 2017), but in contrast to previous studies these authors used different experimental conditions, which include less discriminative epifluorescence microscopy, thicker slices, distinct tissue fixation methods and more heterogeneous bladder sample donors.

Improvement of OAB symptoms by β_3-adrenoceptor agonists, like mirabegron, was initially attributed to direct relaxation of the detrusor smooth muscle (Igawa et al., 1999; Takeda et al., 1999; Wuest et al., 2009), but this assumption has been questioned (Andersson, 2017; Okeke et al., 2017) mostly because the peak plasma levels of mirabegron when administrated in therapeutic (50 mg daily) doses (83-167 nM) are below its potency (EC_{50}~1-3 μM) to relax detrusor smooth muscle strips (Michel and Korstanje, 2016). Newer theories suggest that β_3-adrenoceptor agonists decrease OAB symptoms by reducing afferent nerves activity during bladder filling (Woods et al., 2001; Aizawa et al., 2015) and/or by inhibiting ACh release from parasympathetic postganglionic nerves innervating the detrusor (D' Agostino et al., 2015). Recently, our group added valuable information to these theories by showing that β_3-adrenoceptors-induced inhibition of bladder overactivity may be indirectly mediated by adenosine released from detrusor smooth muscle fibers leading to retrograde activation of inhibitory A_1 receptors localized on cholinergic nerve terminals (Silva et al., 2017). These new paradigms may be clinically relevant because, contrary to the initial belief that normally no parasympathetic nerve drive was observed during bladder storage, recent evidence demonstrates that there is release of ACh from both neuronal and nonneuronal sources, such as the urothelium

and lamina propria, during the filling phase of the micturition cycle (Yoshida et al., 2004; reviewed in Winder et al., 2014). This cholinergic trend may be directly or indirectly exaggerated in overactive bladder patients via the balance between “regulatory” (e.g. noradrenaline, adenosine, NO) and “danger” (e.g. ATP, substance P, prostaglandins) molecules (Birder and de Groat, 2006; Silva et al., 2015; 2017). Reduction of the cholinergic tone responsible for nonvoiding bladder contractions, acting in coordination with the sensory inhibitory drive caused by β_3-adrenoceptor agonists and endogenous adenosine (via A_1 receptors) shown in anaesthetized rats, may increase bladder compliance during the filling phase of the micturition cycle resulting in improvement of OAB symptoms (Silva et al., 2017).

β_3-Adrenoceptors usually couple to adenylyl cyclase via $G_{\alpha s}$ proteins leading to the intracellular accumulation of cAMP (Tyagi et al., 2009). Despite increases in intracellular cAMP have been associated with detrusor relaxation, controversy still exists on whether the prototypical activation of adenylyl cyclase and cAMP generation by β_3-adrenoceptor agonists is the single mechanism responsible for the inhibitory effect of these drugs on urinary bladder tone; other possibilities include modulation of membrane potential, ion-channels activity, and intracellular ion concentrations (Uchida et al., 2005; reviewed in Michel and Vrydag, 2006; Yamaguchi and Chapple, 2007). Notwithstanding this, data from the present study indicate that both isoprenaline (via β_3-adrenoceptors activation) and the receptor-independent adenylyl cyclase catalytic subunit activator, FSK (Seamon et al., 1981), decreased by a similar amount evoked [^{3}H]ACh release from urothelium-denuded detrusor strips. Both drugs also diminished the number and amplitude of non-voiding spontaneous microcontractions and the micturition frequency in the anaesthetized rat, indicating the involvement of the cAMP-dependent pathway downstream β_3-adrenoceptors activation in the urinary bladder.

Intracellular cAMP can be rapidly metabolized into AMP and adenosine by highly effective phosphodiesterases and 5’-nucleotidase, respectively. Consequently, this raises the transmembrane gradient forcing the nucleoside outflow to the extracellular compartment. Imaging and functional studies from our group suggest that β_3-adrenoceptors-induced adenosine release from detrusor bladder strips results from translocation of the nucleoside across the plasma membrane of smooth muscle fibers via the ENT1 transporter subtype (Silva et al., 2017). Here, we show that β_3-adrenoceptors activation with nanomolar concentrations of mirabegron favors the

release of adenosine through a dipyridamole-sensitive ENT in urothelium-denuded human detrusor strips, thus confirming that the mechanism underlying the nucleoside outflow is similar to that observed in the rat bladder (Silva et al., 2017). Cyclic AMP may also be actively transported outside cells via several members of the group of ATP-binding cassette (ABC) transporters (Chen et al., 2001; Guo et al., 2003; Jedlitschky et al., 2000; van Aubel et al., 2002). Even though this mechanism has been described at the urinary bladder (Uchida et al., 2005) and cAMP can originate adenosine in the extracellular space through the sequential hydrolysis by ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1) and ecto-5'nucleotidase/CD73 (see, e.g., Chiavegatti et al., 2008; Sassi et al., 2014), our findings indicate that extracellular cAMP is slowly metabolized into AMP by ENPP1 which makes this pathway very little efficient in the detrusor of both humans and rats (Silva et al., 2017).

Besides contributing to generate intracellular adenosine leading to translocation of the nucleoside to the extracellular milieu, β_3-adrenoceptors-induced accumulation of intracellular cAMP results in downstream activation of cAMP-dependent signalling pathways. The best known cellular effects of cAMP are attributed to the activation of PKA and cyclic nucleotide-regulated ion channels (reviewed in Wang et al., 2017). Activation the cAMP/PKA pathway has been involved in urinary bladder neurotransmission (Losavio and Muchnik, 2000), but no consensus exist on this matter considering the disparity of the results obtained using PKA inhibitors (e.g. H-89) in bladder nerves and detrusor (see, e.g. Frazier et al., 2008). As a matter of fact, inhibition of PKA with H-89 blocked clenbuterol-induced detrusor relaxation while not affecting stimulation-induced muscle tone (Hudman et al., 2000).

Mounting evidence point towards novel cAMP targets to explain cell responses that are insensitive to PKA inhibition (reviewed in Dekkers et al., 2013), which include the exchange protein directly activated by cAMP (EPAC) pathway (de Rooij et al., 1998; Kawasaki et al., 1998). While both PKA and EPAC inhibitors, respectively H-89 and ESI-09, prevented β_3-adrenoceptors-induced release of adenosine in urothelium-denuded detrusor strips from both humans and rats, only the EPAC inhibitor was able to remove the inhibitory effect of β_3-adrenoceptor agonists on evoked [^{3}H]ACh release from human and rat detrusor strips, as well as on the voiding frequency of anaesthetized rats. The ability of ESI-09 to selectively and competitively inhibit EPAC

activity when applied in concentrations below 20 μM has been demonstrated (Zhu et al., 2015) both *in vitro* (Almahariq et al., 2013) and *in vivo* (Gong et al., 2013; Almahariq et al., 2015). Thus, our findings suggest that stimulation of β_3-adrenoceptors favours adenosine outflow leading to inhibition of urinary bladder activity through a mechanism involving preferentially downstream activation of the EPAC pathway in human and rat detrusor samples.

Paradoxical findings (e.g. adenosine outflow vs. ACh release) may result from the fact that PKA and EPAC may act independently, converge synergistically, or oppose each other in regulation of specific cellular functions depending upon their relative abundance, distribution and localization, as well as the specific cellular environments (Cheng et al., 2008). It seems that both pathways, PKA and EPAC, are equally active in the control of ENT1-mediated adenosine release from human and rat detrusor smooth muscle fibers following β_3-adrenoceptors activation with mirabegron and isoprenaline, respectively. Thus, one should expect inhibition of the two cAMP-dependent pathways with H-89 (PKA) and ESI-09 (EPAC) to cause similar preventive effects on β_3-adrenoceptors-induced inhibition of cholinergic nerves activity and of the sensory drive of the micturition reflex, unless PKA exerts a counteracting effect on these neural pathways that opposes its effect on adenosine release from detrusor smooth muscle fibers. Divergent effects of PKA activation within different cell types of the same tissue (e.g. urinary bladder) have been reported before using H-89 as PKA inhibitor (Frazier et al., 2008).

Unlike PKA, EPAC is a cAMP-regulated guanine nucleotide exchange factor (GEF) with no kinase activity that favors GDP/GTP exchange and, thereby, activation of small Ras-like GTPases, such as Rho, Rac and Ras (de Rooji et al., 1998; Kawasaki et al., 1998). Two variants of EPAC protein have been characterized, EPAC1 and EPAC2; both forms are ubiquitously expressed. The expression of EPAC1- and EPAC2-related proteins has been recently demonstrated in the human detrusor smooth muscle (Hayashi et al., 2016). However, their functional role and underlying signaling targets are still unresolved. Here, we raise a novel hypothesis suggesting that the EPAC signaling pathway is the main responsible for the inhibitory effect of β_3-adrenoceptor agonists in the urinary bladder by favouring the release of adenosine from human detrusor smooth muscle fibers via ENT1 transporters. The participation of the EPAC signaling cascade in cAMP-related cell adhesion (Ranjarajan et al., 2003; Enserink et al., 2004), cell communication (Cullere et al.,

2005; Kooistra et al., 2005), cell exocytosis/secretion (Ozaki et al., 2000; Li et al., 2007; Seino et al., 2005; Maillet et al., 2003), cell proliferation, viability and differentiation (Kiermayer et al., 2005; Certal et al., 2015), gene expression and phagocytosis (Cheng et al., 2008) brings up the possibility that some of these processes affecting the pathophysiology and progression of OAB syndromes might also be influenced by therapeutic doses of β_3-adrenoceptor agonists.

Whether differences in the molecular targets of PKA- and EPAC-activated pathways reflect their distinct influence on β_3-adrenoceptors-mediated inhibition of urinary bladder function remains to be elucidated in future studies. Interestingly, the link between adenylyl cyclase activation, intracellular cAMP accumulation and downstream activation of cAMP-dependent pathways may change according to the β-adrenoceptor subtype and probably the pathologic condition. For instance, the β_3-adrenoceptor regulates cystic fibrosis transmembrane conductance regulator chloride current (CFTR), but not the mutated DF508-CFTR, through a PKA-independent pathway (Leblais et al., 1999). On the other hand, β_1- and β_2-adrenoceptors, but not the β_3-adrenoceptor, mediated cAMP-dependent enhancement of vascular endothelial growth factor release by differentiated human monocytic cell challenged with lipopolysaccharide (LPS) through downstream PKA activation (El-Zohairy et al., 2015). Another hypothesis to explain the differential activation of cAMP signalling pathways is the observation that cytosolic cAMP unevenly diffuses within the cell and concentrates in certain local microdomains (Baillie, 2009). Differences in the distribution and activity of intracellular cAMP produced by prostaglandin EP2 and β_2 receptors was demonstrated in human airway smooth cells (Agarwal et al., 2017). Taking into account these complexities, investigation on whether they influence bladder sensitivity to β_3-adrenoceptor drugs due to changes in receptor coupling to distinct downstream cAMP targets, should be undertaken in the context of OAB syndromes. This hypothesis is strengthened because it has been demonstrated that cAMP-dependent regulation of RhoA/Rho-kinase overexpression attenuates detrusor overactivity in an experimental mouse model (Akakpo et al., 2017) and inhibition of this EPAC-regulated kinase can also reverse acetic acid-induced bladder overactivity in rats (Wróbel and Rechberger, 2015).

Interestingly, we show here that β_3-adrenoceptors and adenosine A_1 receptors are overexpressed in detrusor lysates of obstructed BPH patients compared to control men, but the opposite was verified regarding the amount of the ENT1 protein.

CONCLUSION

Downregulation of the ENT1 protein expression in obstructed BPH patients may contribute to restrain adenosine release and, therefore, the A_1 receptor-mediated inhibition of transmitter release (Silva-Ramos et al., 2015a) to the levels one would not expect upon activation of overexpressed β_3-adrenoceptors in the detrusor of BPH patients. Shortage of adenosine due to deficient outflow via ENT1 from smooth muscle fibers might contribute to upregulate A_1 receptors on cholinergic nerves in the detrusor of BPH patients, but limits their full activation capacity resulting in an overall identical inhibitory effect of the β_3-adrenoceptor agonist, mirabegron, on cholinergic neurotransmission among patients and controls. Other authors have shown that mirabegron reduced carbachol-induced myographic contractions in isolated human detrusor preparations from patients with BOO with and without detrusor overactivity and from patients with normal bladder function with a similar potency (SvalØ et al., 2013). BOO has been associated with increased detrusor smooth muscle hypertrophy and bladder fibrosis (Collado et al., 2016). Thus, one may speculate that targeting β_3-adrenoceptors, the cAMP-dependent EPAC signaling pathway and adenosine A_1 receptors may be therapeutically beneficial not only to decrease cholinergic bladder hyperactivity (Silva-Ramos et al., 2015a; Silva et al., 2017), but also to prevent irreversible bladder remodeling secondary to obstruction and/or overactivity profiting from the anti-proliferative role of the EPAC pathway on activated fibroblasts (Certal et al., 2015).

ACKNOWLEDGEMENTS

This research was partially supported by Fundação para a Ciência e a Tecnologia (FCT, FEDER funding, projects PTDC/SAU-OSM/104369/2008, REEQ/1168/SAU/2005, REEQ/1264/SAU/2005, Pest-OE/SAU/UI215/2014 and UID/BIM/4308/2016), Associação Portuguesa de Urologia (APU) and University of Porto/Caixa Geral de Depósitos (Investigação Científica na Pré-Graduação). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. Isabel Silva was in receipt for a PhD Studentship from FCT (SFRH/BD/88855/2012). The authors wish to thank Mrs. Helena Costa e Silva and Belmira Silva for their valuable technical assistance.

VII. GENERAL DISCUSSION

It is widely accepted that purines, in particular ATP, are important regulators of urinary bladder functions in humans, as well as in other animal species. This nucleotide regulates both afferent and efferent signaling pathways. ATP (i) activates sensory nerves during bladder filling conveying both normal and abnormal sensations, like urgency and pain, (ii) acts as a co-transmitter with acetylcholine (ACh) in the parasympathetic control of bladder contraction during voiding, and (iii) participates in the central control of bladder reflexes (Fowler et al., 2008; Birder and Andersson, 2013). The purinergic tone increases significantly with age and it is responsible for up to 40% of nerve-evoked atropine-resistant bladder contractions in pathological conditions, including hypertrophic unstable bladder, overactive detrusor, neurogenic bladder and interstitial cystitis (Burnstock, 2001; Burnstock, 2006). The mechanisms underlying lower urinary tract symptoms (LUTS), as well as overactive bladder (OAB), are largely unknown, but these are usually divided into urothelial, myogenic and neurogenic. It thus appears that ATP plays a chief role in all of these mechanisms. Therefore, one may anticipate benefits in the treatment of LUTS using a combination of drugs targeting the purinome, which include ATP release sites, ectonucleotide-metabolizing enzymes, P1 and P2 purinoceptors and nucleoside transporters, all of these playing relevant roles in regulating urinary bladder function.

Bladder ATP originates from both neuronal and non-neuronal sources, such as urothelial cells, interstitial cells, fibroblast-like cells and smooth muscle fibers. In the urinary bladder the primary source of ATP is the urothelium (Kumar et al., 2004). Associations between increased ATP levels (purinergic signaling) and lower urinary tract disorders have been intensively explored along of the past few years; however, the mechanisms causing these relationships are still unclear. In this context, our group explored the urinary ATP levels in a group of women with overactive bladder (OAB) and men with bladder outlet obstruction (BOO) due to benign prostatic hyperplasia (BPH), compared with age-matched controls. Besides determination of ATP levels in the urine, we also analyzed the content of nerve growth factor (NGF) in the same samples, taking into consideration the reported influence of NGF in the patophysiology of OAB syndromes (Liu et al., 2011; Ochodnicky et al., 2012; Cruz, 2014). Our results show that urinary ATP, but not NGF, normalized by urinary creatinine or voided volume was significantly higher in OAB women with detrusor overactivity than in healthy controls (Silva-Ramos et al., 2013). We also found a

relationship between OAB severity and ATP levels, i.e. patients with OAB wet and low mean voided volume had significantly higher urinary ATP levels. Our results support a role of ATP in the pathogenesis of detrusor overactivity and suggest that urinary ATP may be a highly-sensitive dynamic biomarker of detrusor overactivity in women with OAB syndrome (Silva-Ramos et al., 2013). Non-invasive urinary ATP measurements may represent an improvement compared to other diagnostic and follow-up procedures requiring invasive urodynamic investigations (Silva-Ramos et al., 2013).

LUTS are rather common in adult men over 60 years of age. Although BOO due to benign prostatic hyperplasia (BPH) is a common cause of LUTS, intrinsic bladder dysfunctions and disorders outside the urinary tract can contribute to these symptoms. In Europe, the prevalence of BOO due to BPH in patients with LUTS is about 50% (Oelke et al., 2007; Ockrim et al., 2001). Like in OAB women, our results showed that urinary ATP levels were also significantly higher in patients with BOO due to BPH than in age-matched control men; this was still verified after normalization of urinary ATP levels by the voided volume (Silva-Ramos et al., 2016a). Results suggest that urinary ATP can be a high sensitive non-invasive biomarker of BOO, which may have a discriminative value of detrusor competence when comparing BPH patients with low urinary flow rates (Silva-Ramos et al., 2016a). Our findings are clinically relevant because BPH is a very common disorder, but the differencial diagnosis of low urinary flow due to BOO or to underactive detrusor is difficult in the clinical practice, unless invasive urodynamic tests are performed. This is because LUTS are poor predictors of BOO and symptoms scoring tests alone are insufficient for accurate decision-making diagnosis (de la Rosette et al., 1998). Thus, the possibility of diagnosing detrusor competence in patients with BOO due to BPH using a non-invasive biochemical test is appealing, which makes urinary ATP a highly-sensitive dynamic biomarker of BOO (Silva-Ramos et al., 2016a). In a rat model of partial BOO, it was demonstrated that bladder obstruction affects both urothelial and sub-urothelial cells leading to increases in ATP release from these cells (Shiina et al., 2016). These authors suggested that increased extracellular ATP levels may motivate non-voiding bladder tension and cause bladder instability. Besides many other signaling molecules released from the urothelium (e.g. neurotrophins, acetylcholine, prostaglandins, nitric oxide and cytokines), ATP

seems to be the main contributor to the voiding reflex and bladder pain (Merril et al., 2016).

The role of ATP as potential biomarker of bladder diseases is corroborated animal models of overactive and underactive bladder dysfunctions (Munoz et al., 2011). ATP was used to follow the benefits of BoNT-A for the treatment of OAB in an animal model of chronic spinal cord injury (Smith et al., 2008). It is a common belief that investigation of the mechanisms underlying the activity of biomarkers migh contribute to a better understanding of the pathophysiology of certain diseases, like OAB syndromes (Antunes-Lopes et al., 2014). All evidence demonstrate that ATP has a great impact in LUTS, strengthenig our opinion that investigation of the bladder "purinome" may be paramount to prompt for novel therapeutic alternatives to common medications used in the treatment of OAB syndromes.

Contrasting to the compelling evidence about the role of ATP and the purinergic cascade in bladder function and dysfunction, the role of uracil nucleotides in autocrine/paracrine actions controling bladder activity has increasingly been reported. Both, uridine triphosphate (UTP) and uridine diphosphate (UDP) were detected in human urine samples (Contreras-Sanz et al., 2012). Similarly to ATP, the amount of uracil nucleotides was also increased in the urine of OAB patients (Contreras-Sanz et al., 2012). Using urethane-anaesthetized rats, our group showed that activation of UDP-sensitive urothelial $P2Y_6$ receptors augmented the voiding frequency indirectly by increasing the release of ATP from urothelial cells (Carneiro et al., 2014). Bladder overactivity produced by urothelial activation of $P2Y_6$ receptors depends on the activation of ATP-sensitive P2X3 and P2X1 receptors located on sub-urothelial nerve afferents and smooth muscle detrusor fibers, respectively. Fine-tuning control of this excitatory bladder drive may result from ATP hydrolysis to ADP by NTPDase2 located in the lamina propria (probably on interstitial cells) and on cholinergic nerve efferents. Formation of ADP at the cholinergic neuromuscular synapse decreases ACh release and, subsequent, detrusor contraction through the activation of prejunctional inhibitory $P2Y_1$ receptors in rats (Carneiro et al., 2014).

Deregulation of purinergic bladder signaling may contribute to persistent detrusor overactivity in patients with BOO. Work performed in the context of this thesis showed that activation of $P2Y_6$ receptors also facilitates non-neuronal ATP and ACh release from the urothelium of men with BOO due to BPH. These findings led us to propose selective $P2Y_6$ receptor blockade as a novel therapeutic strategy to

control persistent bladder storage symptoms in obstructed patients. The positive feedback involving the participation of ATP-sensitive P2X3 (and/or P2X2/3) receptors in the mechanosensory purinergic drive commanding the micturition reflex found in anaesthetized rats (Sun and Chai, 2006; Carneiro et al., 2014) was also observed in the urothelium of control men, but not in preparations of BPH patients. The lack of effect of fast desensitizing ATP-sensitive receptors might be explained because, in contrast to $P2Y_6$, the immunoreactivity against P2X2 and P2X3 receptors decreased significantly in the urothelium of BPH patients (see also, Ruggieri, 2006). Urothelial ATP release may be a specific modulator in native tissue by purinergic and muscarinic neurotransmitters via distinct mechanisms. Released ATP produces paracrine effects on underlying tissues (Siu et al., 2014).

Recently, the pharmacological profiles of spontaneous contractions of the mucosa and detrusor layers of the bladder wall were characterized separately. Interestingly, the magnitude of spontaneous contractions was higher in the intact bladder, followed by the isolated mucosa and the detrusor layer in that ordeer. This is paradoxical if one considers the percentage of smooth muscle fibers compared to other cell types, which was greater in the detrusor than in the mucosa (Kushida and Fry, 2016). Comparing mucosa and detrusor, the pharmacological profiles of the spontaneous contractions induced by P2X and P2Y receptor agonists, adenosine and capsaicin, were also different. Intact preparations showed tension responses which were intermediate to those obtained in the isolated mucosa and detrusor. Low extracellular pH generated bigger changes in the detrusor than in the mucosa. Mucosal preparations released ATP in an oscillatory manner following variations in the spontaneous contractions. The ATP release was greater in the mucosa compared to the detrusor and it was augmented by carbachol and reversed by the M_2-selective antagonist, methoctramine (Kushida and Fry, 2016). Differences in the pharmacological profiles between the bladder mucosa and detrusor, implies distinct mechanisms underlying contractile activation. Also, the intermediate responses obtained from intact preparations imply a dynamic interaction between the two bladder layers. The temporal relationship between ATP release oscillations and the amplitude of spontaneous contractions is consistent with ATP release controlling the bladder spontaneous activity (Kushida and Fry, 2016).

Despite the differences in the purinergic control of urothelial function, we show here that the ATP/ACh release ratio was fivefold higher in the mucosa of obstructed

BPH patients than in control individuals. Impairment of the extracellular ATP catabolism by NTPDases may account for this difference (Harvey et al., 2002; Silva-Ramos et al., 2015a), along with a partial loss of choline acetyltransferase (ChAT) which together with the mitochondrial enzyme carnitine acetyltransferase are responsible for synthesizing ACh in the urothelium (Lips et al., 2007).

The mechanisms that regulate the release of ATP in the urinary bladder are still a matter of debate, but may include vesicle exocytosis (Nakagomi et al., 2016), translocation via pannexin-1 containing hemichannels (Shout et al., 2002; Timóteo et al., 2014; Negoro et al., 2013), transient receptor potential channels, like TRPV1 (Charrua et al., 2009) and TRPV4 (Mochizuki et al., 2009), Piezo1 channels (Miyamoto et al., 2014), among others. Our findings show that facilitation of ATP release from urothelial cells triggered by $P2Y_6$ receptor agonists was prevented by pannexin-1 hemichannel inhibitors both in rats (Timóteo et al., 2014) and the same was observed in the human samples. Co-localization of pannexin-1 and $P2Y_6$ receptors in the rat urothelium strengthens the functional data implicating pannexin-1 as a major releasing pathway of ATP following $P2Y_6$ receptors activation (Timóteo et al., 2014).

Storage of ATP in synaptic vesicles of cholinergic nerves is known for many years (reviewed in Mutafova-Yambolieva and Durnin, 2014). Whether this also applies to the urothelium is a matter of debate. In 1997, Fergusson and collaborators raised three argument levels against the vesicular release of ATP from the urothelium: i) rather than inhibiting ATP release, removal of Ca^{2+} from the extracellular medium actually potentiated the release of the nucleotide, ii) tetrodotoxin, applied in concentrations that fully blocked nerve-evoked detrusor contractions had no significant effect on electrically-evoked ATP from the urothelium, and iii) although the suburothelial sensory nerves are packed with secretory granules, there are no such granules inside urothelial cells (Fergusson et al., 1997). On the other hand, McLatchie and Fry (2014) stimulated urothelial cells in suspension by imposing upon them a mild drag force stress and found that urothelial ATP release was reduced in low (1.8 mM) calcium medium, but it increased roughly by two-fold upon increasing intracellular calcium (McLatchie and Fry, 2014). Under these circumstances urothelial ATP release was also reduced by agents blocking pannexin and connexin hemichannels. The calcium-dependence of ATP release and

its influence by connexin / pannexin channel blockers suggested that a major fraction (up to 50%) of the nucleotide release is through these hemichannels (McLatchie and Fry, 2014). However, the conspicuous effect of *N-ethylmaleimide,* which has been proposed to reduce vesicular docking to the surface membrane of secretory cells, is consistent with a substantial fraction of release by vesicular exocytosis (Andersson, 2015). Thus, further characterization of the pathways involved in urothelial ATP release may help to develop new therapeutic strategies for disorders assumed to be characterised by increased ATP release, such as overactive bladder syndromes.

Further studies are still required to elucidate the complexity of the interplay between various P2 purinoceptors to control non-neuronal reflexes in the human bladder mucosa, both in health and disease conditions. Extracellular ATP accumulation may contribute to the loss of P2X2 and P2X3 receptors immunostaining and function in the urothelium of BPH patients. Excess of extracellular ATP released from the urothelium in response to stretch and chemical irritants may contribute to hyperexcitation of suburothelial nerve afferents via homomeric P2X3 and/or heteromeric P2X2/3 receptors. In contrast to that observed in the urothelium, increased levels of ATP released by the mucosa of OAB patients is associated with an overexpression of P2X3 and/or heteromeric P2X2/3 receptors on suburothelial nerves fibers.

Using immunofluorescence confocal microscopy, we showed that NTPDases1 and 2 are deficient in obstructed BPH patients, while ecto-5´nucleotidase is fairly conserved in both groups. This result is compatible with our findings showing a significant reduction of about 1.4 and 1.9 in the kinetics of ATP and ADP catabolism in these patients compared to control individuals, which result in deficient adenosine formation from the extracellular catabolism of adenine nucleotides despite no differences were observed in ecto-5'nucleotidase expression and activity (Silva-Ramos et al., 2015a). Impairment of NTPDases activity has been described in other disorders, including those affecting the central nervous system, such as schizophrenia (SZ). Reduction in striatal NTPDase activity may contribute to the pathophysiology of SZ, which might explain the deficits in adenosine signaling detected in this illness (Aliagas et al., 2013).

The slower extracellular ADP inactivation compared to ATP in urothelium-denuded detrusor preparations of BPH patients, combined with the presence of

NTPDase2 on VAChT-positive cholinergic nerve terminals (Carneiro et al., 2014), might favour transient ADP accumulation at the cholinergic neuromuscular synapse and activation of presynaptic ADP-sensitive receptors. To test the activity of these receptors we used the enzymatically-stable ADP analogue, ADPβS and selective $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ receptor antagonists. Our results indicate that drugs that favour ADP accumulation in the cholinergic neuromuscular synapse of the human detrusor and/or that promote activation of overexpressed inhibitory $P2Y_{12}$ receptors may be a suitable therapeutic strategy to counteract cholinergic bladder activity associated with obstruction in BPH patients. Data also show that blockade of fast desensitizing homomultimeric P2X3 and heteromultimeric P2X2/3 ionotropic receptors may additionally be used to cease bladder overacitivity by acting on both sides (sensory and motor, respectively) of the micturition reflex (see also, Takezawa et al., 2017).

So far, our data suggest the use of selective $P2Y_6$ receptor antagonists to decrease the excessive sensory bladder drive triggered by ATP (and ACh) release from injured urothelial cells due to BOO associated with BPH. Whether this theory also applies to surplus ATP release from the urothelium of women with OAB, requires experimental validation using bladder samples that are much more difficult to obtain. Our data also prompted to the beneficial effects of $P2Y_{12}$ receptor agonists and/or P2X2/3 receptor antagonists in order to restrain hyperactivity of cholinergic bladder nerves in obstructed patients. Alternatively, blockade of P2X3 receptors on suburothelial sensory bladder nerves, with drugs like AF-742, is already are being considered for the treatment of OAB and bladder pain (Burnstock, 2011; Sacco et al., 2015). Interestingly, therapeutic interventions using “purinome-based strategies” have also been advanced for interstitial cystitis. In this context it has been hypothesized the use of adenosine A_1 receptor antagonists, like DPCPX, and P2X7 receptor antagonists (Aronsson et al., 2012).

The therapeutic success of β_3-adrenoceptor agonists, like mirabegron, for managing OAB syndromes has generated a great interest in the discovery of their mechanism of action. It has been suggested that stimulation of β_3-adrenoceptors improve OAB symptoms by directly relaxing detrusor smooth muscle contractions as a consequent of intracellular cyclic AMP (cAMP) formation. Increasing evidence suggest distinct, eventually more therapeutically relevant, mechanisms of action of

β_3-adrenoceptors, which includes an inhibitory effect on nerve-evoked ACh release (D'Agostino et al., 2007). However, the presence of β_3-adrenoceptors on bladder cholinergic nerve terminals has been disputed (Coelho et al., 2017). Consensus exists more on the predominant localization of β_3-adrenoceptors on detrusor smooth muscle fibers (Igawa et al., 1998, 1999).

In this thesis, we tested a newer theory to explain the inhibitory effect of β_3-adrenoceptor agonists on cholinergic bladder transmission, which considered the following premises: (i) adenosine A_1 receptors present on cholinergic nerves of the human detrusor downregulate ACh release (Silva-Ramos et al., 2015a), and (ii) adenosine may originate from cAMP catabolism, in a process that depends on the sequential activity of ecto-nucleotidase/phosphodiestase 1 (ENPP1) and ecto-5´nucleotidase/CD73 (Chievegatti et al., 2008; Sassi et al., 2014). Our hypothesis was that adenosine formed from the catabolism of cAMP in the detrusor may act as a retrograde messenger via prejunctional A_1 receptors to explain inhibition of cholinergic activity by β_3-adrenoceptor agonists. Data show that β_3-adrenoceptors activation decreases electrically-evoked [^{3}H]ACh release from human and rat detrusor strips indirectly by favoring adenosine outflow from smooth muscle fibers via equilibrative nucleoside transporters (ENT) and activation of inhibitory A_1 receptors localized on cholinergic nerve terminals. This mechanism involves adenylyl cyclase activation and intracellular cAMP accumulation because it was mimicked by FSK, which directly activates the catalytic subunit of adenylyl cyclase without the need for receptor couping to G_s proteins. Interestingly, both FSK and β_3-adrenoceptor agonists also significantly decreased the amplitude and frequency of non-voiding contractions, as well as the voiding frequency, in urethane-anaesthetized rats.

Besides being considered a source of adenosine, intracellular cAMP accumulation following β_3-adrenoceptors activation triggers a series of downstream signaling events which are not fully characterized in the urinary bladder. The involvement of cAMP-dependent PKA activation downstream G_s-protein-coupled receptors activation, like the β_3-adrenoceptor, has been a matter of debate in the literature mostly because PKA inhibitors produce divergent effects (see e.g. Frazier et al., 2005). Increasing evidence point towards the participation of cAMP-activated ion channels and the exchange protein directly activated by cAMP (EPAC) to explain cAMP-dependent effects that are resistant to PKA inhibition (de Rooij et al., 1998;

Kawasaki et al., 1998). Our results show clearly that β_3-adrenoceptors promote adenosine release, reduce evoked [^{3}H]ACh release and decrease the voiding frequency through a mechanism that is dependent on downstream activation of the EPAC, but not PKA, signaling pathway both in human and rat urinary bladders. The link between β_3-adrenoceptors stimulation and downstream EPAC activation leading to increases in adenosine outflow via the ENT1 transporter subtype remains to be investigated.

Thus, β_3-adrenoceptor agonists with the participation of endogenous adenosine may exert inhibitory effects on bladder function increasing the storage capacity and prolonging the micturition interval, without affecting the voiding pressure or post-void residual volume. In this context, it remains to be explored the role of β_3-adrenoceptor agonists on urothelium-derived adenosine released from mechanically-stimulated umbrella cells, which are also endowed with ENT1 nucleoside transporters. Our findings led us to propose a novel mechanism involving cAMP-mediated activation of EPAC, endogenous adenosine release and A_1 receptors activation in the urinary bladder which contributes to explain, at least partially, the therapeutic success of β_3-adrenoceptor agonists on OAB symptoms. These drugs may act both (1) on the sensory bladder drive operated by mechanically-sensitive urothelial cells sensory nerve afferents and interstitial pacemaker cells, and (2) on the efferent motor component resulting in the reduction of non-voiding cholinergic microcontractions and in the increase in bladder storage capacity by relaxing the detrusor. Apparently, these processes are effectively cut-short during the voiding command by yet unknown mechanisms, which may involve the unrestrained parasympathetic boost. As a matter of fact, β_3-adrenoceptor agonists do not affect voiding urodynamic parameters (maximum urinary flow and detrusor pressure at maximal urinary flow) in clinical settings and we did not observed any changes in the duration and amplitude of voiding urinary contraction in response to isoprenaline in the anaesthetized rat, nor these were parameters affected by blockage of adenosine A_1 receptors with DPCPX. Here we clearly demonstrate that selective activation of β_3-adrenoceptors with mirabegron is equally active to control cholinergic neurotransmission in the urinary bladder of obstructed BPH patients. This was verified despite β_3-adrenoceptors and adenosine A_1 receptors are overexpressed in the detrusor of BPH patients, which emphasizes the limiting role exerted by

downregulated ENT1 nucleoside transporter on adenosine tonus at the bladder cholinergic synapse. In view of this, we propose that pharmacological manipulation of endogenous adenosine levels and/or A_1 receptor activation may act synergistically with β_3-adrenoreceptors activation to control bladder overactivity in these patients.

VIII. REFERENCES

Abbracchio MP, Burnstock G, Boeynaems J-M, Barnard EA, Boyer JL, Kennedy C, Knight G, Fumagalli M, Gachet C, Jacobson K, Weisman GA. International Union of Pharmacology LVIII: update on the P2Y G-protein-coupled nucleotide receptors: from molecular mechanism and pathophysiology to therapy. Pharmacol Rev. 2006. 58(3):281-341.

Abbracchio MP, Burnstock G, Verkhratsky A, Zimmermann H. Purinergic signaling in the nervous system: an overview. Trends Neurosc. 2009. 32: 19-29.

Abbracchio MP, Burnstock G. Purinergic signaling: pathophysiological roles. J Pharmacol. 1998. 78: 113-145.

Abbracchio MP, Burnstock G. Purinoceptors: are there families of P2X and P2Y purinoceptors? Pharmacol Therap. 1994. 64: 445-475.

Abrams P, Kaplan S, De Koning Gans HJ, Millard R. Safety and tolerability of tolterodine for the treatment of overactive bladder in men with bladder outlet obstruction. J Urol. 2006a. 175: 999-1004.

Abrams P, Andersson KE, Buccafusco JJ, Chapple C, de Groat WC, Fryer AD, Kay G, Laties A, Nathanson NM, Pasricha PJ, Wein AJ. Muscarinic receptors: their distribution and function in body systems, and the implications for treating overactive bladder. Br J Pharmacol. 2006b. 148: 565-78.

Abrams P, Cardozo L, Fall M, Griffiths D, Rosier P, Ulmsten U, van Kerrebroeck P, Victor A, Wein A. The standardization of terminology of lower urinary tract function: report from the standardisation sub-committee of the International Continence Society. Neurourol Urodyn. 2002. 21: 167-78.

Abrams P. New words for old: lower urinary tract symptoms for “prostatism”: BMJ. 1994. 308: 929-30.

Acevedo CG, Contreras E, Escalona J, Lewin J, Huidobro-Toro JP. Pharmacological characterization of adenosine A1 and A2 receptors in the bladder: evidence for a modulatory adenosine tone regulating non-adrenergic non-cholinergic neurotransmission. Br J Pharmacol. 1992. 107: 120-126.

Acevedo CG, Contreras E. Possible involvement of adenine nucleotides in the neurotransmission of the mouse urinary bladder. Comp Biochem Physiol. 1985. 82: 357-361.

Ahlquist RP. A study of the adrenotropic receptors. Am J Physiol. 1948. 153: 586-600.

Aizawa N, Homma Y, Igawa Y. Effects of L-arginine, mirabegron, and oxybutynin on the primary bladder afferent nerve activities synchronized with reflexic, rhythmic bladder contractions in the rat. Neurourol Urodyn. 2015. 34: 368-374.

Akakpo W, Misicki B, Burnett A. cAMP-Dependent regulation of RhoA/Rho-kinase attenuates detrusor overactivity in a novel mouse experimental model. BJU Int. 2017. 120 (1): 143-151.

Akino H, Chapple CR, Makay N, Cross RL, Murakami S, Yokayamma O, Chess-Williams R, Sellers DJ. Spontaneous contractions of the pig urinary bladder: the effect of ATP-sensitive potassium channels and the role of mucosa. BJU Int. 2008. 102: 1168-1174.

Ali H, Müller CE, Daly JW, Beaven MA. Methylxanthines block antigen-induced responses in RBL-2H3 cells independently of adenosine receptors or cyclic AMP: evidence for inhibition of antigen binding to IgE. J Pharmacol Exp Ther. 1991. 258(3): 954-62.

Almahariq M, Mei FC, Wang H, Cao AT, Yao S, Soong L, Sun J, Cong Y, Chen J, Cheng X. Exchange Protein Directly Activated by cAMP (EPAC1) Modulates Regulatory T Cell-Mediated Immune Suppression. Biochem J. 2015. 465: 295–303.

Almahariq M, Tsalkova T, Mei FC, Chen H, Zhou J, Sastry SK, Schwede D, Cheng X. A novel EPAC-specific inhibitor suppresses pancreatic cancer cell migration and invasion. Mol Pharmacol. 2013. 83: 122–128.

Amark P, Nergårdh A, Kinn AC. The effect of noradrenaline on the contractile response of the urinary bladder. An in vitro study in man in cat. Scan J Urol Nephrol. 1986. 20(3): 203-7.

Andersson KE, Arner A. Urinary bladder contraction and relaxation: physiology and pathophysiology. Physiol Rev. 2004. 84(3): 935-86.

Andersson KE, Campeau L, Olshansky B. Cardiac effects of muscarinic receptor antagonists used for voiding dysfunction. Br J Clin Pharmacol. 2011. 72: 186-96.

Andersson KE, Martin N, Nitti V. Selective β3-adrenoceptors agonists for the treatment of overactive bladder. J Urol. 2013. 190: 1173-8.

Andersson KE, Persson K. Nitric oxide synthase and the lower urinary tract: Possible implications for physiology and pathophysiology. Scan J urol Nephrol. 1995. 175:43-55.

Andersson KE. New developments in the management of overactive bladder: focus on mirabegron and onabotulinumtoxin A. Ther Clin Risk Manag. 2013. 9: 161-70.

Andersson KE. Pharmacology of lower urinary tract smooth muscles and penile erectile tissues. Pharmacol Rev. 1993. 45: 253-308.

Andersson KE. Pharmacology: On the mode of action of mirabegron. Nat Rev Urol. 2016. 13: 131-132.

Andersson KE. Potential future pharmacological treatment of bladder dysfunction. Basic Clin Pharmacol Toxicol. 2016. 119 (Suppl 3): 75-85.

Angulo JC, Khullar V, Nitti VW, Siddigui E. Evidence available on the use of the selective β3-adrenoceptor agonist mirabegron for the treatment of overactive bladder. Actas Urol Esp. 2013. 37(10): 640-651.

Apodaca G. The uroepithelium: Not just a passive barrier. Traffic. 2004. 5:117–128.

Applebaum AE, Vance WH, Coggeshall RE. Segmental localization of sensory cells that innervate the bladder. J Comp Neurol. 1980. 192: 203-209.

Aronsson P, Andersson M, Ericsson T, Giglio D. Assessment and Characterization of Purinergic Contractions and Relaxations in the rat urinary bladder. Basic & Clinic Pharmacol & Toxicology. 2010. 107: 603-613.

Auli M, Martinez E, Gallego D, Opazo A, Espin F, Marti-Gallostra M, Jiménez M, Clavé P. Effect of excitatory and inhibitory neurotransmission on motor patterns of human sigmoid colon in vitro. Br J Pharmacol. 2008. 155: 1043-1055.

Baillie GS. Compartmentalized signaling: spatial regulation of cAMP by the action of compartmentalized phosphodiesterases. FEBS J. 2009. 276:1790–99.

Baker JG. The selectivity of beta-adrenoceptor agonists at human β1-, β2- and β3- adrenoceptors. Br J Pharmacol. 2010. 160: 1048-1061.

Barendrecht MM, Frazier EP, Vrydag W, Alewijnse AE, Peters SL, Michel MC. The effect of bladder outlet obstruction on alpha1- and beta-adrenoceptor expression and function. Neurourol Urodyn. 2009. 28: 349-355.

Beckel J, Birder LA. Differential expression and function of nicotinic acetylcholine receptors in the urinary bladder epithelium of the rat. J Physiol. 2012. 590(6): 1485-1480.

Beckel J, Holstege G. Neurophysiology of the Lower Urinary Tract. Hand Book Exp Pharmacol. 2011. 202: 149-69.

Beckel J, Holstege G. The Rat Nervous System – 4th edition; Chapter 12: The Lower Urinary tract", edited by George Paxinos. 2015.

Beckel JM, Kanai A, Lee SJ, de Groat WC, Birder LA. Expression of functional nicotinic acetylcholine receptors in rat urinary bladder epithelial cells. Am J Physiol Renal Physiol. 2006. 290: 103-110.

Bharucha AE. Pelvic floor: Anatomy and function. Neurogastroenterol Motil. 2006. 18: 507-519.

Birder LA, Andersson KE. Urothelial signaling. Physiol Rev. 2013. 93: 653-680.

Birder LA, Apodaca G, de Groat WC, Kanai AJ. Adrenergic and capsaicin-evoked nitric oxide release from urothelium and afferent nerves in urinary bladder. Am J Physiol. 1998. 275:F226 – F229.

Birder LA, Barrick SR, Roppolo JR. Feline interstitial cystitis results in mechanical hypersensitivity and altered ATP release from bladder urothelium. Am J Physiol Renal Physiol. 2003. 285: F423-F9.

Birder LA, de Groat WC. Mechanisms of disease: involvement of the urothelium in bladder dysfunction. Nat Clin Pract Urol. 2007. 4:46-54.

Birder LA, Hanna-Mitchell AT, Mayer E, Buffington CA. Cystitis, co-morbid disorders and associated epithelial dysfunction. Neurourol Urodyn. 2011. 30(5): 668-72.

Birder LA, Kanai AJ, de Groat WC, Kiss S, Nealen ML, Burke NE, Watkins S, Reynolds IJ, Caterina MJ. Vanilloid receptor expression suggests a sensory role for urinary bladder epithelial cells. Proc Natl Acad Sci USA. 2001. 98:13396–13401.

Birder LA, Nealen ML, Kiss S, de Groat WC, Caterina MJ, Wang E, Apodaca G, Kanai AJ. Beta-adrenoceptor agonists stimulate endothelial nitric oxide synthase in rat urinary bladder urothelial cells. J Neurosci 2002, 22: 8063-8070.

Birder LA, Ruan HZ, Chopra B, Xiang Z, Barrick S, Buffington CA, Roppolo JR, Ford AP, de Groat WC, Burnstock G. Alterations in P2X and P2Y purinergic receptor expression in urinary bladder from normal cats and cats with interstitial cystitis. Am J Physiol Renal Physiol. 2004. 287: F1084-F1091.

Birder LA. Nervous network for lower urinary tract function. Int J Urol. 2013. 20:4-12.

Birder LA. Urinary bladder urothelium: Molecular sensors of chemical/ thermal/ mechanical stimuli. Vasc Pharmacol. 2006. 45: 221-226.

Birder LA. Urothelial signaling. Handb Exp Pharmacol. 2011. 202: 207–231.

Birder LA; Ruan HZ, Chopra B, Xiang Z, Barrick S, Buffington CA, Roppolo JR, Ford AP, de Groat WC, Burnstock G. Alterations in P2X and P2Y purinergic receptor expression in urinary bladder from normal cats and cats with interstitial cystitis. Am J Physiol Renal Physiol. 2004. 287(5): F1084-91.

Boeynaems JM, Communi F, Gonzalez NS, Robaye B. Overview of the P2 receptors. Semin Thromb Hemost. 2005. 31: 139-149.

Boeynaems JM, Wilkin F, Marteau F, Duhant X, Savi P, Gonzalez NB, Robaye B, Communi D. P2Y receptors: new subtypes, new functions. Drug Dev Res. 2003. 59: 30-35.

Bollen M, Gijsbers R, Ceulemans H, Stalmans W, Stefan C. Nucleotide pyrophosphatase/phosphodiesterases on the more. Crit Rev Biochem Mol Biol. 2000. 35: 393-432.

Bolton TB, Zholos AV. Activation of M2 muscarinic receptors in guinea-pig ileum opens cationic channels modulated by M3 muscarinic receptors. Life Sci. 1997. 60: 1121-1128.

Bonner TI, Buckley NJ, Young AC, Brann MR. Identification of a family of muscarinic acetylcholine receptor genes. Science. 1987. 237: 527-532.

Bonner TI, Young AC, Brann MR, Buckley NJ. Cloning and expression of the human and rat M5 muscarinic acetylcholine receptor genes. Neuron. 1988. 1: 403-410.

Bonner TI. New subtypes of muscarinic acetylcholine receptors. Trends Pharmacol Sci. 1989b. 11-15.

Bonner TI. The molecular basis of muscarinic receptor diversity. Trends Neurosci. 1989a. 12: 148-151.

Braas KM, May V, Zvara P, Nausch B, Kliment J, Dunleavy JD, Nelson MT, Vizzard MA. Role of pituitary adenylate cyclase activating polypeptide in cystitis-induced plasticity of micturition reflexes. Am J Physiol Regul Integr Comp Physiol. 2006. 290 (4): R951-R962.

Brady CM, Apostolidis A, Yiangou Y, Baecker PA, Ford AP, Freeman A, Jacques TS, Flower CJ, Anand P. P2X3-immunoreactive nerve fibres in neurogenic detrusor overactivity and the effect of intravesical resiniferatoxin. Eur Urol. 2004. 46: 247-253.

Brake AJ, Wagenbach MJ, Julius D. New structural motif for ligand-fated ion channels defined by an ionotropic ATP receptor. Nature. 1994. 371: 519-523.

Brederson JD, Jarvis MF. Homomeric and heteromeric P2X3 receptors in peripheral sensory neurons. Curr Opin Investig Drugs. 2008. 9: 716-725.

Bridgeman MB, Friia NJ, Taft C, Shah M. Mirabegron: β3-adrenergic receptor agonist for the treatment of overactive bladder. Ann Pharmacother. 2013. 47: 1029-38.

Brown C, Burnstock G, Cocks T. Effects of adenosine 5'-triphosphate (ATP) and beta-gamma-methylene ATP on the rat urinary bladder. Br J Pharmacol. 1979. 65: 97-102.

Bschleipfer T, Schukowski K, Weidner W, Grando SA, Schwantes W, Kummer W, Lips KS. Expression and distributions of cholinergic receptors in the human urothelium. Life Sciences. 2007. 80: 2303-2307.

Burnstock G, Cocks T, Crowe R, Kasakov L. Purinergic innervation of the guinea-pig urinary bladder. Br J Pharmacol. 1978. 63:125-138.

Burnstock G, Cocks T, Kasakov L, Wong HK. Direct evidence for ATP release from non-adrenergic, non-cholinergic "purinergic" nerves in the guinea-pig taenia coli and bladder. Eur J Pharmacol. 1978. 49: 145-149.

Burnstock G, Dumsday B, Smythe A. Atropine resistant excitation of the urinary bladder: the possibility of transmission via nerves releasing a purine nucleotide. Br J Pharmacol. 1972. 44: 451-461.

Burnstock G, Knight GE. Cellular distribution and functions of P2 receptor subtypes in different systems. Int Rev Cytol. 2004. 240: 31-304.

Burnstock G. A basis for distinguishing two types of purinergic receptor. In Cell Membrane Receptors for Drugs and Hormones: A Multidisciplinary Approach (Straub RW and Bolis L, eds), Raven Press. 1978. 107-118.

Burnstock G. Historical review: ATP as a neurotransmitter. Trends in Pharmacol Sci. 2006. 27 (3).

Burnstock G. Noradrenaline and ATP: cotransmitters and neuromodulators. J Physiol Pharmacol. 1995. 46: 365-384.

Burnstock G. P2X receptors in sensory neurons. Br J Anaesth. 2000. 84(4): 476-88.

Burnstock G. Pathophysiology and therapeutic potential of purinergic signaling. Pharmacol Rev. 2006. 58:58-86.

Burnstock G. Physiology and pathophysiology of purinergic neurotransmission. Physiol Rev. 2007. 87(2): 659-797.

Burnstock G. Purinergic nerves. Pharmacol Rev. 1972. 24: 509-581.

Burnstock G. Purinergic signaling in lower urinary tract. In Handbook of Experimental Pharmacology, Vol 151 (I). Purinergic and Pyrimidinergic Signalling I – Molecular, Nervous and Urinogenitary System Function (Abbracchio MP and Williams M, eds), Springer-Verlag. 2001: 423-515.

Burnstock G. Purinergic signaling in the urinary tract in health and disease. Purinerg Signal. 2013. 10(1): 103-155.

Burnstock G. Purinergic signaling. BJ Pharmacol. 2006. 147: S172-S181.

Burnstock G. Purinergic signaling: Therapeutic developments. Front Pharmacol. 2017. 8: 661.

Burnstock G. Purinoceptors: ontogeny and phylogeny. Drug Dev Res. 1996. 39: 204-242.

Burnstock G. Release of vasoactive substances from endothelial cells by shear and purinergic mechanosensory transduction. J Anat. 1999. 194: 335-342.

Bylund DB, Eikenberg DC, Hieble JP (1994) IV. International Union of Pharmacology Nomenclature of Adrenoceptors. Pharmacol Rev, 46: 121-36.

Calvert RC, Thompson CS, Burnstock G. ATP release from the human ureter on distension and P2X3 receptor expression on suburothelial sensory nerves. Purinergic Sign. 2008. 4: 377-381.

Candelore MR, Deng L, Tota L, Guan XM, Amend A, Liu Y, Newbold R, Cascieri MA, Weber AE. Potente and selective human β3-adrenergic receptor antagonists. J Pharmacol Exp Ther. 1999. 290: 649-655.

Carneiro I, Timóteo MA, Silva I, Vieira C, Baldaia C, Ferreirinha F, Silva-Ramos M, Correia-de-Sá P. Activation of $P2Y_6$ receptors increases the voiding frequency in anaesthetized rats by releasing ATP from the bladder urothelium. Br J Pharmacol. 2014. 174(14): 3404-19.

Carrière I, Fourrier-Reglat A, Dartigues JF, Rouaud O, Pasquier F, Ritchie K, Ancelin ML. Drugs with anticholinergic properties, cognitive decline, and dementia in an elderly general population. Arch Intern Med. 2009. 169(14): 1317-1324.

Carter RL, Fricks IP, Barrett MO, Burianek LE, Zhou Y, Ko H, Das A, Jacobson KA, Lazarowski ER, Harden TK. Quantification of Gi-mediated inhibition of

adenylyl cyclase activity reveals that UDP is a potent agonist of the human $P2Y_{14}$. Mol Pharmacol. 2009. 76(6): 1341-8.

Caudfield MP, Birdsall NJM. International Union of Pharmacology. XVII. Classification of muscarinic acetylcholine receptors. Pharmacol Rev. 1998. 50: 279-290.

Cernecka H, Ochodnicky P, Lamers WH, Michel MC. Specificity evaluation of antibodies against human β3-adrenoceptors. Nauncy Schmiedeberg's Arch Pharmacol. 2012. 385: 875-882.

Certal M, Vinhas A, Pinheiro AR, Ferreirinha F, Barros-Barbosa AR, Silva I, Costa MA, Correia-de-Sá P. Calcium signaling and the novel anti-proliferative effect of the UTP-sensitive $P2Y_{11}$ receptor in rat cardiac myofibroblasts. Cell Calcium. 2015. 58(5): 518-33.

Chancellor MB, Fowler CJ, Apostolidis A, de Groat WC, Smith CP, Somogyi GT, Aoki KR. Drug Insight: biological effects of botulinum toxin A in the lower urinary tract. Nat Clin Pract Urol. 2008. 5(6): 319-28.

Chancellor MB, Kaplan SA, Blaivas JG. The cholinergic and purinergic components of detrusor contractility in a whole rabbit bladder model. J Urol. 1992. 148: 906-909.

Chapple C, Khullar V, Gabriel Z, Muston D, Bitoun CE, Weinstein D. The effects of antimuscarinic treatments in overactive bladder: an update of a systematic review and meta-analysis. Eur Urol. 2008. 54: 543-62.

Chapple C. Pathophysiology of the lower urinary tract and CNC. Can Urol Assoc. 2011. 5(Suppl 2): S126-7.

Charrua A, Reguenga C, Cordeiro JM, Correia-de-Sá P, Paule C, Nagy I, Cruz F, Avelino A. Functional transient receptor potential vanilloid 1 is expressed in human urothelial cells. J Urol. 2009. 182(6): 2944-50.

Chen ZS, Lee K, Kruh GD. Transport of cyclic nucleotides and estradiol 17-β-D-glucuronide by multidrug resistance protein 4. Resistance to 6-mercaptopuurine and 6-thioguanine. J Biol Chem. 2001. 276: 33747-33754.

Cheng X, Ji Z, Tsalkova T, Mei F. Epac and PKA: a table of two intracellular cAMP receptors. Acta Biochem Biophys Sin. 2008. 40(7): 651-662.

Cheng Y, Mansfield KJ, Allen W, Chess-Williams R, Burcher E, Moore KH. ATP during early bladder stretch is important for urgency in detrusor overactivity patients. Biomed Res Int. 2014. 2014: 204604.

Cheng Y, Mansfield KJ, Allen W, Millard RJ, Burcher E, Moore KH. Correlation between cystometric volumes, ATP release, and pH in women with overactive bladder versus controls. Neurourol Urodyn. 2013. 32(7): 969-73.

Cheng Y, Mansfield KJ, Allen W, Walsh CA, Burcher E, Moore KH. Does adenosine triphosphate released into voided urodynamic fluid contribute to urgency signaling in women with bladder dysfunction? J Urol. 2010. 183(3): 1082-6.

Cheng Y, Mansfield KJ, Sandow SL, Sadananda P, Burcher E, Moore KH. Porcine bladder urothelial, myofibroblasts, and detrusor muscle cells: characterization and ATP release. Front Pharmacol. 2011. 7: 2-27.

Chess-Williams R, Chapple CR, Yamanishi T, Sellers DJ. The minor population of M3 receptors mediate contraction of human detrusor muscle in vitro. J Auton Pharmacol. 2001. 21: 243-248.

Chiavegetti T, Costa VL Jr, Araújo MS, Godinho RO. Skeletal muscle expresses the extracelular cyclic AMP-adenosine pathway. Br J Pharmacol. 2008. 153:1331-1340.

Cho KJ, Koh JS, Choi J, Kim JC. Changes in adenosine triphosphate and nitric oxide in the urothelium of benign prostate hyperplasia in patients with detrusor underactivity. J Urol. 2017. 17: 76743-2.

Chopra B, Barrick SR, Meyers S, Beckel JM, Zeidel ML, Ford APDW, de Groat WC, Birder LA. Expression and function of bradykinin B1 and B2 receptors in normal and inflamed rat urinary bladder urothelium. J Physiol. 2005. 562(3): 859-871.

Chopra B, Gever J, Barrick SR, Hanna-Mitchell AT, Beckel JM, Ford AP, Birder LA. Expression and function of rat urothelial P2Y receptors. Am J Physiol Renal Physiol. 2008. 294(4): F821-F829.

Chou EC, Capello SA, Levin RM, Longhurst PA. Excitatory α1-adrenergic receptors predominate over inhibitory β-receptors in rabbit dorsal detrusor. J Urol. 2003. 170: 2503-2507.

Chua ME, Lapitan MCM, Silangcruz JMA, Jr Luna S, Jr Morales ML. Beta-3 adrenergic receptor agonist for adult with overactive bladder. Cochrane database Syst Rev. 2015. (3): 1-9.

Cockayne DA, Dunn PM, Zhong Y, Rong W, Hamilton SG, Knight CE, Ruan HZ, Ma B, Yip P, Nunn P, McMahon SB, Burnstock G, Ford AP. P2X2 knockout mice and P2X2/P2X3 double knockout mice reveal a role for the P2X2 receptor subunit in mediating multiple sensory effect of ATP. J Physiol. 2005. 567: 621-639.

Cockayne DA, Hamilton SG, Zhu QM, Dunn PM, Zhong Y, Novakovic S, Malmberg AB, Cain G, Berson A, Kassotakis L, Hedley L, Lachnit WG, Burnstock G, McMahon SB, Ford AP. Urinary bladder hyporeflexia and reduced pain-related behavior in P2X3-deficient mice. Nature. 2000. 407: 1011-1015.

Coelho A, Antunes-Lopes T. Gillespie J, Cruz F. Beta-3 adrenergic receptor is expressed in acetylcholine- containing nerve fibers of the human urinary bladder: An immunohistochemical study. Neurourol Urodyn. 2017. 36(8): 1972-1980.

Collado A, Batista E, Gelabert-Más A, Corominas JM, Arañó P, Villavicencio H. Detrusor quantitative morphometry in obstructed males and controls. J Urol. 2006. 176: 2722-8.

Coman OA, Păunescu H, Ghită I, Coman L, Bădărău A, Fulga I. Beta3-adrenergic receptors: molecular, histological, functional and pharmacological approaches. Rom J Morph Embry. 2009. 50(2):169-179.

Communi D, Gonzalez NS, Detheux M, Brézillon S, Lamoy V, Parmentier M, Boeynaems JM. Identification of a novel human ADP receptor coupled to G(i). J Biol Chem. 2001. 276(44): 41479-85.

Contreras-Sanz A, Schott-Ward TS, Gill HS, Jacoby JC, Birch RE, Malone-Lee J, Taylor KM, Peppiatt-Wildman CM, Wildman SS. Simultaneous quantification of 12 different nucleotides and nucleosides released from renal epithelium and in human urine samples using ion-pair reversed-phase HPLC. Purinergic Signal. 2012. 8(4): 741-51.

Correia S, Dinis P, Rolo F, Lunet N. Prevalence, treatment and known risk factors of urinary incontinence and overactive bladder in the non-institutionalized Portuguese population. Int Urogyn. 2009. 20: 1481-1489.

Correia-de-Sá P, Adães S, Timóteo MA, Vieira C, Magalhães-Cardoso T, Nascimento C, Duarte-Araújo M. Fine-tuning modulation of myenteric motoneurons by endogenous adenosine: on the role of secreted adenosine deaminase. Auton Neurosci. 2006. 126-127: 211-224.

Correia-de-Sá P. Pathophysiological significance of nucleotide hydrolyzing enzymes in urologic disorder. Purinergic Signal. 2010. 6(Suppl 2), S13.13.

Cowan WD, Daniel EE. Human female bladder and its noncholinergic contractile function. Can J Physiol Pharmacol. 1983. 61(11): 1236-46.

Cullere X, Shaw SK, Andersson L, Hirahashi J, Luscinskas FW, Mayadas TN. Regulation of vascular endothelial barrier function by Epac, a cAMP-activated exchange factor for Rap GTPase. Blood. 2005. 105:1950–1955.

Cunha RA, Sebastião AM, Ribeiro JA. Ecto-5´-nucleotidase is associated with cholinergic nerve terminals in the hippocampus but not in the cerebral cortex of the rat. J Neurochem. 1992. 59(2): 657-66.

Cunha RA. Adenosine as a neuromodulator and has a homeostatic regulator in the nervous system: different roles, different sources and different receptors. Neurochem Int. 2001. 38(2): 107-25.

Currie CJ, McEwan P, Poole CD, Odeyemi IA, Datta SN, Morgan CL. The impact of the overactive bladder on health-related utility and quality of life. BJU Int. 2006. 97: 1267-72.

D'Agostino G, Condino AM, Calvi V, Boschi F, Gioglio L, Barbieri A. Purinergic P2X3 heteroreceptors enhance parasympathetic motor drive in isolated porcine detrusor, a reliable model for development of P2X selective blockers for detrusor hyperactivity. Pharmacol Res. 2012. 65: 129-136.

D'Agostino G, Condino MA, Calvi P. Involvement of β_3-adrenoceptors in the inhibitory control of cholinergic activity in human bladder: Direct evidence by [^{3}H]-acetylcholine release experiments in the isolated detrusor. Eur J Pharmacol. 2015. 758: 115-122.

D'Mello R, Dickenson AH. Spinal cord mechanisms of pain. Br J Anaesth. 2008. 101: 8-16.

Daly DM, Chess-Williams R, Chapple C, Grundy D. The inhibitory role of acetylcholine and muscarinic receptors in bladder afferent activity. Eur Urol. 2010. 58: 22-8.

De Biasi M, Nigro F, Xu W. Nicotinic acetylcholine receptors in the autonomic control of bladder function. Eur J Pharmacol. 2000. 393: 173-140.

de Groat WC, Booth AM, Yoshimura N. Neurophysiology of micturition and its modification in animals models of human disease. In: The Autonomic Nervous System. Nervous Control of the Urogenital System, ed. by Maggi CA, Hartwood Academic, London. 1993. 3: 227-289.

de Groat WC, Booth AM. Nervous Control of the Urogenital System (Autonomic Nervous System). Ed. Maggi CA. Vol. 3. Harwood Academic Publishers; London. 1993. 9:291-347.

de Groat WC, Booth AM. Physiology of the urinary bladder and urethra. Ann Intern Med. 1980. 92: 312-315.

de Groat WC, Lalley PM. Reflex firing in the lumbar sympathetic outflow to activation of vesical afferent fibers. J Physiol. 1972. 226: 289-309.

de Groat WC, Yoshimura N. Changes in afferent activity after spinal cord injury. Neurourol Urodyn. 2010. 29:63–76.

de Groat WC. Integrative control of the lower urinary tract: preclinical perspective. Br J Pharmacol. 2006. 147 (Suppl 2):S25-40.

de Groat WC. The urothelium in overactive bladder: passive bystander or active participant? Urol. 2004. 64: 4-11.

de la Rosette JJ, Witjes WP, Schafer W, Abrams P, Donovan JL, Peters TJ, Millard RJ, Frimodt-Moller C, Kalomiris P. Relationships between lower urinary tract symptoms and bladder outlet obstruction: results from the ICS- “BPH” study. Neurourol Urody. 1998. 17(2): 99-108.

de Rooij J, Zwartkruis FJ, Verheijen MH, Coll RH, Nijman SM, Wittinghofer A, Bos JL. Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. Nature. 1998. 396: 474–477.

Dickenson JM, Blank JL, Hill SJ. Human adenosine A1 receptor and P2Y2-purinoceptor-mediated activation of the mitogen-activated protein kinase cascade in transfected CHO cells. Br J Pharmacol. 1998. 124(7): 1491-9.

Dixon AK, Gubitz AK, Sirinathsinghji DJ, Richardson PJ, Freeman TC. Tissue distribution of adenosine receptor mRNAs in the rat. Br J Pharmacol. 1996. 118: 1461-1468.

Donaldson SH, Picher M, Boucher RC. Secreted and cell-associated adenylate kinase and nucleoside diphosphokinase contribute to extracellular nucleotide metabolism on human airway surfaces. Am J Respir Cell Mol Biol. 2002. 26: 209-15.

Dorsam RT, Kunapuli SP. Central role of the P2Y12 receptor in platelet activation. J Clin Invest. 2004. 113: 340-5.

Downie JW, Karmazyn M. Mechanical trauma to bladder epithelium liberates prostanoids which modulate neurotransmission in rabbit detrusor muscle. J Pharmacol Exp Ther. 1984. 230:445–449.

Drake MJ, Fowler CJ, Griffiths D, Mayer E, Paton JF, Birder L. Neural control of the lower urinary and gastrointestinal tracts: Supraspinal CNS mechanisms. Neurousol Urodyn. 2010. 29: 119-127.

Drake MJ, Nitti VW, Ginsberg DA, Brucker BM, Hepp Z, McCool R, Glanville JM, Fleetwood K, James D, Chapple C. Comparative assessment of the efficacy of onabotulinumtoxinA and oral therapies (anticholinergics and mirabegron) for overactive bladder: a systematic review and network meta-analysis. BJU Int. 2017. 120: 611-622.

Drake R, Vogl W, Mitchell AWM, Tibbitts R, Richardson P. Gray's Atlas of Anatomy (4th Edi.) Edinburgh: Churchill Livingstone. 2008.

Duarte-Araújo M, Nascimento C, Timóteo MA, Magalhães-Cardoso T, Correia-de-Sá P. Dual effects of adenosine on acetylcholine release from myenteric motoneurons are mediated by junctional facilitatory A2A and extrajunctional inhibitory A1 receptors. Br J Pharmacol. 2004. 141: 925-934.

Duarte-Araújo M, Timóteo MA, Correia-de-Sá P. Adenosine activation A_{2A}-receptors coupled to adenylate cyclase/cyclic AMP pathway downregulates nicotinic autoreceptor function at the rat myenteric nerve terminals. Neurochem Int. 2004. 45: 641-651.

Dunning-Davies BM, Fry CH, Mansour D, Ferguson DR. The regulation of ATP release from the urothelium by adenosine and transepithelial potential. BJU Int. 2013. 111: 505-513.

El Hachmane MF, Komai AM, Olofsson CS. Cooling reduces cAMP-stimulated exocytosis and adiponectin secretion at a Ca2+ - dependent step in 3T3-L1 adipocytes. Plos One. 2015. 10:e0119530.

Elbadawi A. neuromuscular mechanism of micturition. In: Yalla SV, ME Elbadawi A. Eds, Neurology and Urodynamics: Principle and Practice. Macmillan, New York. 1988: 3-36.

Elgoyhen AB, Vetter DE, Katz E, Rothlin CV, Heinemann SF, Boulter J. α10: a determinant of nicotinic cholinergic receptor function in mammalian vestibular and cochlear mechanosensory hair cells. Proc Nat Sci USA. 2001. 98: 3501-3506.

Elneil S, Skepper JN, Kidd EJ, Williamson JG, Ferguson DR. Distribution of P2X(1) and P2X(3) receptors in the rat and humans urinary bladder. Pharmacology. 2001. 63(2): 120-8.

El-Zohairy SN, Oriowo MA, Ezeamuzie CI. Cyclic adenosine monophosphate-mediated enhancement of vascular endothelial growth factor released by differentiated human monocytic cells: the role of protein kinase A. Med Princ Pract. 2015. 24:548-554.

Enserink JM, Price LS, Methi T, Mahic M, Sonnenberg A, Bos JL, Taskén K. The cAMP-Epac-Rap1 pathway regulates cell spreading and cell adhesion to laminin-5 through the alpha3beta1 integrin but not the alpha6beta4 integrin. J Biol Chem. 2004. 279:44889–44896.

Evans BA, Papaioannou M, Hamilton S, Summers RJ. Alternative splicing generates two isoforms of the beta3-adrenoceptor which are differentially expressed in mouse tissues. Br J Pharmacol. 1999. 127: 1525-1531.

Fasciani I, Temperán A, Pérez-Atencio LF, Escudero A, Martínez-Montero P, Molano J, Gómez-Hernández JM, Paino CL, González-Nieto D, Barrio LC. Regulation of connexin hemichannel activity by membrane potential and the extracellular calcium in health and disease. Neuropharmacology. 2013. 75: 479-90.

Ferguson DR, Kennedy I, Burton TJ. ATP is released from rabbit urinary bladder epithelial cells by hydrostatic pressure changes - A possible sensory mechanism? J Physiol. 1997. 505:503–511.

Floyd K, Hick VE, Morrison JF. Mechanosensitive afferent units in the hypogastric nerve of the cat. J Physiol. 1976. 259: 457-471.

Ford AP, Gever JR, Nunn PA, Zhong Y, Cefalu JS, Dillon MP, Cockayne DA. Purinoceptors as therapeutic targets for lower urinary tract dysfunction. Br J Pharmacol. 2006. 147 (Suppl 2): S132-43.

Ford AP, Undem BJ. The therapeutic promise of ATP antagonism at P2X3 receptors in respiratory and urological disorders. Front Cell Neurosci. 2013. 7: 267.

Ford AP. In pursuit of P2X3 antagonists: novel therapeutics for chronic pain and afferent sensitization. Purin Sign. 2012. 8 (Suppl1): 3-26.

Fowler CJ, Griffiths D, de Groat WC. The neural control of micturition. Nat Rev Neurosci. 2008. 9(6): 453-466.

Fowler CJ. Integrated control of lower urinary tract – clinical perspective. Br J Pharmacol. 2006. 147: S14-S24.

Frazier EP, Mathy MJ, Peters SL, Michel MC. (2005). Does cyclic AMP mediate rat urinary bladder relaxation by isoproterenol? J Pharmacol Exp Ther. 2005. 313(1), 260–7.

Frazier EP, Peters SL, Braverman AS, Ruggieri MRSr, Michel M. Signal transduction underlying the control of urinary bladder smooth muscle tone by muscarinic receptors and beta-adrenoceptors. Nauny Schm Arch Pharmacol. 2008. 377(4-6): 449-62.

Fredholm BB, Arslan G, Halldner L, Kull B, Schulte G, Adén U, Svenningsson P. Adenosine receptor signaling in vitro and in vivo. Drug Dev Res. 2001. 52: 274-282.

Fredholm BB, IJzerman AP, Jacobson KA, Klotz KN, Linden J. International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. Pharmacol Rev. 2001. 53: 527-552.

Fredholm BB, IJzerman AP, Jacobson KA; Linden J, Müller CE. International Union of Basic and Clinical Pharmacology. LXXXI. Nomenclature and classification of adenosine receptors-an update. Pharmacol Rev. 2011. 63(1): 1-34.

Fredriksson R, Lagerstrom MC, Lundin LG, Schioth HB. The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. Mol Pharmacol. 2003. 63: 1256-1272.

Freire VS, Burkhard FC, Kessler TM, Kuhn A, Draeger A, Monastyrskaya K. MicroRNAs may mediate the dwnregulation of neurokinin-1 receptor in chronic bladder pain syndrome. Am J Path. 2010. 176 (1): 288-303.

Frias B, Lopes T, Pinto R, Cruz F, Cruz CD. Neurotrophins in the lower urinary tract: becoming of age. Curr Neuropharmacol. 2011. 9(4): 553-8.

Fry C, Young J. The physiology and pharmacology of the lower urinary tract. Surgery (Oxford) Bas Sci. 2010. 28 (7): 317-322.

Fry C. Physiology of the lower urinary tract. Surgery (Oxford) Renal and Urology. 2008. 35 (9) 26:4.

Fry CH, Kanai AJ, Roosen A, Takeda M, Wood DN. Incontinence. Abrams P, Cardozo L, Khoury S, Wein A, editors. Health Publications Ltd; Jersey. 2005: 313-362.

Fry CH, Sui GP, Kanai AJ, Wu C. The function of suburothelial myofibroblasts in the bladder. Neurourol Urodyn. 2007. 26:914–919.

Fry CH, Young HS, Jabr RI, McCarthy C, Ikeda Y, Kanai AJ. Modulation of spontaneous activity in the overactive bladder: the role of P2Y agonists. Am J Physiol Renal Physiol. 2012. 302: F1447.

Fuhrich DG, Lessey BA, Savaris RF. Comparison of HSCORE assessment of endometrial beta 3 integrin subunit expression with digital HSCORE using computerized image analysis (ImageJ). Anal Quant Cytopathol Histopathol. 2013. 35: 210-6.

Fujimura R, Tamura K, Tsutsumi T, Yamamoto T, Nakamura K, Koibuchi Y, Kobayashi M, Yamaguchi O. Expression and possible functional role of the beta3-adrenoceptor in human and rat detrusor muscle. J Urol. 1999. 161: 680-685.

Fumagalli M, Trincarelli L. Lecca D, Martini C, Ciana P, Abbracchio MP. Cloning, pharmacological characterization and distribution of the rat G-protein-coupled $P2Y_{13}$ receptor. Biochem Pharmacol. 2004. 68: 113-124.

Gachet C, Hechler B. The platelet P2 receptors in thrombosis. Semin Thromb Hemost. 2005. 31: 162-167.

Giglio D, Ryberg AT, To K, Delbro DS, Tobin G. Altered muscarinic receptor subtype expression and functional responses in cyclophosphamide induced cystitis in rats. Auton Neurosc: Basic and Clin. 2005. 122 (1-2): 9-20.

Gijsbers R, Aoki J, Arai H, Bollen M. The hydrolysis of lysophospholipids and nucleotides by autotaxin (NPP2) involves a single catalytic site. FEBS Lett. 2003. 538: 60-64.

Gillespie JI, Rouget C, Palea S, Granato Cm Korstanje C. Beta adrenergic modulation of spontaneous microcontractions and electrical field-stimulated contractions in isolated strips of rat urinary bladder from normal animals and animals with partial bladder outflow obstruction. Naunyn Schmiedeberg's Arch Pharmacol. 2015. 388: 719-26.

Goding JW, Howard MC. Ecto-enzymes of lymphoid cells. Immunol Rev. 1998. 161: 5-10.

Gong B, Shelite T, Mei FC, Ha T, Hu Y, Xu G, Chang Q, Wakamiya M, Ksiazek TG, Boor PJ, Bouyer DH, Popov VL, Chen J, Walker DH, Chens X. Exchange protein directly activated by cAMP plays a critical role in bacterial invasion during fatal rickettsioses. Proc Natl Acad Sci USA. 2013. 110: 19615–19620.

Gonzalez EJ, Heppner TJ, Nelson MT, Vizzard MA. Purinergic signaling underlies transforming growth factor-β-mediated bladder afferent nerve hyperexcitability. J Physiol. 2016. 594(13): 3575-88.

Gonzalez EJ, Merrill L, Vizzard MA. Bladder sensory physiology: neuroactive compounds and receptors, sensory transducers, and target-derived growth factors as

targets to improve function. Am J Physiol Regul Integr Comp Physiol. 2014. 306: R869-R878.

Gopalakrishnan SM, Buckner SA, Milicic I, Groebe DR, Whiteaker KL, Burns DJ, Warrior U, Gopalakrishnan M. Functional characterization of adenosine receptors and coupling to ATP-sensitive K+ channels in Guinea pig urinary bladder smooth muscle. J Pharmacol Exp Ther. 2002. 300 (3): 910-7.

Gosling JA, Dixon JS. Sensory nerves in the mammalian urinary tract. An evaluation using light and electron microscopy. J Anat. 1974. 117:133–144.

Griffin CS, Bradley E, Hollywood MA, McHale NG, Thornbury KD, Sergeant GP. β3-adrenoceptor agonists inhibit carbachol-evoked Ca^{2+} oscillations in murine detrusor myocytes. BJU Int. 2017. Dec 6 [Epub ahead of print].

Griffiths D, Tadic SD. Bladder control, urgency, and urge incontinence: Evidence from functional brain imaging. Neurourol Urodyn. 2008. 27: 466-474.

Grol S, van Koeveringe GA, de Vente J, van Kerrebroeck PE, Gillespie JI. Regional differences in sensory innervation and suburothelial interstitial cells in the bladder neck and urethra. BJU Int. 2008. 102:870–877.

Guan N, Gustafsson LE, Svennersten K. Inhibitory effects of urothelium-related factors. Basic Clin Pharmacol Toxicol. 2017. 121 (4): 220-224.

Guarracino JF, Cinali AR, Fernández V, Roquel LI, Losavio AS. $P2Y_{13}$ receptors mediate presynaptic inhibition of acetylcholine release induced by adenine nucleotides of the mouse neuromuscular junction. Neurosc. 2016. 326: 31-44.

Guo Y, Kotova E, Chen ZS, Lee K, Hopper-Borge E, Belinsky MG, Kruh GD. MRP8, ATP-binding cassete C11 (ABCC11), is a cyclic nucleotide effluc pump and a resistance factor for fluoropyrimidines 2',3'-dideoxycytidine and 9'-(2'-phosphonymethoxythyl) adenine. J Biol Chem. 2003. 278: 29509-29514.

Habler HJ, Janig W, Koltzenburg M. Activation of unmyelinated afferent fibres by mechanical stimuli and inflammation of the urinary bladder in the cat. J Physiol. 1990. 425:545–562.

Harris S, Rizzolo D. Botulinum toxin as a treatment for refractory overactive bladder. JAAPA. 2016. 29(2): 1-4.

Harvey RA, Skennerton DE, Newgreen D, Fry CH. The contractile potency of adenosine triphosphate and ecto-adenosine triphosphatase activity in guinea pig detrusor and detrusor from patients with a stable, unstable or obstructed bladder. J Urol. 2002. 168: 1235-1239.

Harvey RD, Belevych AE. Muscarinic regulation of cardiac ion channels. Br J Pharmacol. 2003. 139: 1074-84.

Hashitani H, Brading AF, Suzuki H. Correlation between spontaneous electrical, calcium and mechanical activity in detrusor smooth muscle of the guinea-pig bladder. Br J Pharmacol. 2004. 141(1): 183-93.

Hatanaka T, Ukai M, Watanabe M, Somega A, Ohtake A, Suzuki M, Ueshima K, Sato S, Sasamata M. In vitro and in vivo pharmacology profile of the selective β_3-adrenoceptor agonist mirabegron in rats. N-S Arch Pharmacol. 2013. 386: 247-253.

Hayashi M, Kajioka S, Itsumi M, Takahashi R, Shahab N, Ishigami T, Takeda M, Masuda N, Yamaguchi A, Naito S. Actions of cAMP on calcium sensitization in human detrusor smooth muscle contraction. BJU Int. 2016. 117(1): 179-91.

Hegde SS, Choppin A, Bonhaus D, Briaud S, Loeb M, Moy TM, Loury D, Eglen RM. Functional role of M2 and M3 muscarinic receptors in the urinary bladder of rats in vitro and in vivo. Br J Pharmacol. 1997. 120: 1409-18.

Hegde SS, Eglen RM. Muscarinic receptor subtypes modulating smooth muscle contractility in the urinary bladder. Life Sci. 1999. 64: 419-428.

Helfand BT, Evans RM, McVary KT. A comparison of the frequencies of medical therapies for overactive bladder in men and women: analysis of more than 7.2 million aging patients. Eur Urol. 2010. 586-591.

Herbert JM, Savi P. P2Y12, a new platelet ADP receptor, target of clopidogrel. Semin Vasc Med. 2003. 3(2): 113-22.

Hicks M. The mammalian urinary bladder: an accomodating organ. Biol Rev. 1975. 50:215–246.

Hirsh AJ, Stonebraker JR, van Heusden CA, Lazarowski ER, Boucher RC, Picher M. Adenosine deaminase 1 and concentrative nucleoside transporters 2 and 3 regulate adenosine on the apical surface of human airway epithelia: implications for inflammatory lung diseases. Biochemistry. 2007. 46: 10373-10383.

Hoffmann C, Leitz MR, Oberdorf-Maass S, Lohse MJ, Klotz K-N. Comparative pharmacology of human β-adrenergic receptor subtypes – characterization of stably transfected receptors in CHO cells. Naunyn-Schmiedeberg's Arch Pharmacol. 2004. 369: 151-159.

Holstege G, Mounton LJ. Central nervous system control of micturition. Int Rev Neurobiol. 2003. 56: 123-145.

Holstege G. Micturition and the soul. J Comp Neurol. 2005. 493: 15-20.

Holstege G. The emotional motor system and micturition control. Neurourol Urodyn. 2010. 29: 42-48.

Holz GG, Kang G, Harbeck M, Roe MW, Chepurny OG. Cell physiology of cAMP sensor Epac. J Physiol. 2006. 577: 5-15.

Honda M, Takenaka A, Inone S, Chancellor MB, Yoshimura N. Sensory neurone-specific receptor-mediated regulation of micturition reflex in urethane-anaesthetized rats. BJU Int. 2012. 109: 628-633.

Hoyle CH, Chapple C, Burnstock G. Isolated human bladder: evidences for a dinucleotide acting on P2X-purinoceptors and for purinergic transmission. Eur J Pharmacol. 1989. 174(1): 115-8.

Hoyle HV, Burnstock G. Postganglionic efferent transmission in the bladder and urethra. In Nervous Control of the Urogenital System (Maggi Ca Ed), Harwood, Academic Publishers GmbH, Chur, Switzerland. 1993: 349-381.

Hudman D, Elliott RA, Whitaker P, Terry TR, Sandhu DP, Norman RI. Inhibition of the contractile response of isolated human and rat bladders by clenbuterol. J Urol. 2001. 166: 1969-1973.

Husted S, Sjögren C, Andersson KE. Direct effects of adenosine and adenine nucleotides on isolated human urinary bladder and their influence on electrically induced contractions. J Urol. 1983. 130, 392-398.

Igawa Y, Michel MC. Pharmacological profile of beta-3-adrenoceptor agonists in clinical development for the treatment of overactive bladder syndrome. Naunyn Schmiedeberg's Arch Pharmacol. 2013. 386: 177-183.

Igawa Y, Yamazaki Y, Takeda H, Akahane M, Ajisawa Y, Yanoyama T, Nishizawa O. Possible β3-adrenoceptor-mediated relaxation of the human detrusor. Acta Physiol Scand. 1998. 164: 117-118.

Igawa Y, Yamazaki Y, Takeda H, Hayakawa K, Akahane M, Ajisawa Y, Yoneyama T, Nishizawa O, Andersson KE. Functional and molecular biological evidence for a possible beta3-adrenoceptor in the human detrusor muscle. Br J Pharmacol. 1999. 126: 819-825.

Igawa Y, Yamazaki Y, Takeda H, Kaidoh K, Akahane M, Ajisawa Y, Yoneyama T, Nishizawa O, Andersson KE. Relaxant effects of isoproterenol and selective beta3-adrenoceptor agonists on normal, low compliant and hyperreflexic human bladders. J Urol. 2001. 165: 240-244.

Igawa Y, Zhang X, Nishizawa O, Umeda M, Iwata A, Taketo MM, Manabe T, Matsui M, Andersson KE. Cystometric findings in mice lacking muscarinic M2 or M3 receptors. J Urol. 2004. 172: 2460-4.

Irwin DE, Milsom I, Hunskaar S, Reilly K, Kopp Z, Herschorn S, Coyne K, Kelleher C, Hampel C, Artibani W, Abrams P. Population-based survey of urinary incontinence, overactive bladder and other lower urinary tract symptoms in five countries: results if the EPIC study. Eur Urol. 2006b. 50(6): 1306-1315.

Irwin DE, Milsom I, Kopp Z, Abrams P, Cardozo L. Impact of overactive bladder symptoms on employment, social interactions and emotional wellbeing in six European countries. BJU Int. 2006a. 97: 96-100.

Ito K, Iwami A, Katsura H, Ikeda M. Therapeutic effects of the putative P2X3/P2X2/3 antagonist A-317491 on cyclophosphamide-induced cystitis in rats. N-S Arc Pharmacol. 2008. 372:483-490.

Jacobson KA, Costanzi S, Joshi BV, Besada P, Shin DH, Ko H, Ivanov AA, Mamedova L. Agonists and antagonists for P2 receptors. Novartis Found Symp. 2006. 276: 58-68.

Jacobson KA, Jarvis MF, William SM. Purine and pyrimidine (P2) receptors as drug targets. J Med Chem. 2002. 45: 4057-4093.

James G, Butt AM. P2Y and P2X purinoceptors mediated Ca2+ signaling in glial cell pathology in the central nervous system. Eur J Pharmacol. 2002. 447(2-3): 247-60.

Jarvis MF, Burgard EC, McGaraughty S, Honore P, Lynch K, Brennan TJ, Sibieta A, Van Biesen T, Cartmell J, Bianchi B, Niforatos W, Kage K, Yu H, Mikusa J, Wismer CT, Zhu Cz, Chu K, Lee CH, Stewart AO, Polakwaski J, Cox BF, Kowaluk E, Williams M, Sullivan J, Faltynek C. A-317491, a novel potent and selective non-nucleotide antagonist of P2X3 and P2X2/3 receptors, reduces chronic inflammatory and neuropathic pain in the rat. Proc Natl Acad Sci USA. 2002. 99: 17179-17184.

Jedlitschky G, Burchell B, Keppler D. The multidrug resistance protein 5 functions as an ATP dependent export pump for cyclic nucleotides. J Biol Chem. 2000. 275: 30069-30074.

Jen PY, Dixon JS, Gosling JA. Immunohistochemical localization of neuromarkers and neuropeptides in human fetal and neonatal urinary bladder. B J Urol. 1995. 75:230–235.

Jenes Á, Ruzsnavszky F, Telek A, Szigeti GP, Csernoch L. A possible role of the cholinergic and purinergic receptor interaction in the regulation of the rat urinary bladder function. J Muscle Res Cell Motil. 2012. 32: 421-431.

Jhang JF, Kuo HC. Botulinum toxin A and lower urinary tract dysfunction: pathophysiology and mechanisms of action. Toxins (Basel). 2016. 8(4): 120.

Jones SV, Heilman CJ, Brann MR. Functional responses of cloned muscarinic receptors expressed in CHO-K1 cells. Mol Pharmacol. 1991. 40: 242-247.

Jost SP, Gosling JA, Dixon JS. Themorphology of normal human bladder urothelium. J Anat. 1989. 167: 103–115.

Jóźwiak-Bebenista M, Wiktorowska-Owczarek A, Kowalczyk E. Beta-adrenoceptor mediated cyclic AMP signal in different types of culture nerve cells in normoxic and hypoxic conditions. Mol Biol. 2016. 50(5): 838-846.

Kaan TKY, Yip PK, Grist J, Cefalu JS, Nunn PA, Ford AP, Zhong Y, McMahon SB. Endogenous purinergic control of bladder activity via presynaptic P2X3 and P2X2/3 receptors in the spinal cord. Neurosc. 2010. 30(12): 4503-4507.

Kanai A, Andersson KE. Bladder afferent signaling: recent findings. J Urol. 2010. 183(4): 1288-1295.

Kang SH, Chess-Williams R, Anoopkumar-Dukie S, McDermott C. Induction of inflammatory cytokines and alteration of urothelial ATP, acetylcholine and prostaglandin E2 release by doxorubicin. Eur J Pharmacol. 2013. 700: 102-9.

Kaplan SA, Roehrborn CG, Chancellor M, Carlsson M, Bavendam T, Guan Z. Extended-release tolterodine with or without tamsulosin in men with lower urinary tract symptoms and overactive bladder: effects on urinary symptoms assessed by the International Prostate Symptoms Score. BJU Int. 2008. 102: 1133-9.

Kasakov L, Burnstock G. The use of the slowly degradable analoge alpha,beta-methylene ATP, to produce desensitization of the P2-purinoceptor: effect on non-adrenergic, non-cholinergic responses of the guinea-pig urinary bladder. Eur J Pharmacol. 1982. 86: 291-294.

Kavia RB, Dasgupta R, Fowler CJ. Functional imaging and the central control of the bladder. J Comp Neurol. 2005. 493: 27-32.

Kawasaki H, Springett GM, Mochizuki N, Toki S, Nakaya M, Matsuda M, Housman DE, Graybiel AM. A family of cAMP-binding proteins that directly activate Rap1. Science. 1998. 282: 2275–2279.

Kelleher CJ, Cardozo LD, Khullar V, Salvatore S. A medium-term analysis of the subjective efficacy of treatment for women with detrusor instability and low bladder compliance. Br J Obstet Gyneacol. 1997. 104: 988-93.

Kennedy C, Tasker P, Gallacher G, Westfall TD. Identification of atropine- and P2X1 receptor antagonist –resistant, neurogenic contractions of the urinary bladder. J Neurosc. 2007. 27(4): 845:851.

Kessler TM, Bachmann LM, Minder C, Löhrer D, Umbehr M, Schünemann HJ, Kessels AGH. Adverse event assessment of antimuscarinics for treating overactive bladder: a network meta-analytic approach. Plos One. 2011. 6: e16718.

Khera M, Somogyi GT, Kiss S, Boone TB, Smith CP. Botulinum toxin A inhibits ATP release from bladder urothelium after chronic spinal cord injury. Neurochem Int. 2004. 45(7): 987-93.

Kiermayer S, Biondi RM, Imig J, Plotz G, Haupenthal J, Zeuzem S, Piiper A. Epac activation converts cAMP from a proliferative into a differentiation signal in PC12 cells. Mol Biol Cell. 2005.16:5639–5648.

Kim JC, Yoo JS, Park EY, Hong SH, Seo SI, Hwang TK. Muscarinic and purinergic receptor expression in the urothelium of rats with detrusor overactivity induced by bladder outlet obstruction. BJU Int. 2007. 101: 371-375.

King BF, Knowles ID, Burnstock G, Ramage AG. Investigation of the effects of P2 purinoceptor ligands on the micturition reflex in female urethane-anaesthetized rats. Br J Pharmacol. 2004. 142(3): 519-30.

King JA, Huddart H, Staff WG. Purinergic modulation of rat urinary bladder detrusor smooth muscle. Gen Pharmacol. 1997. 29: 597-604.

Kitta T, Chancellor MB, de Groat WC, Kuno S, Nonomura K, Yoshimura N. Suppression of bladder overactivity by adenosine A2A receptor antagonist in a rat model of Parkinson disease. J Urol. 2012. 187(5): 1890-7.

Kitta T, Chancellor MB, de Groat WC, Kuno S, Nonomura K, Yoshimura N. Roles of adenosine A1 and A2A receptors in the control of micturition in rats. Neurourol Urodyn. 2014. 33(8): 1259-65.

Klinger M, Freissmuth M, Nanoff C. Adenosine receptors: G protein-mediated signaling and the role of accessory proteins. Cell Signal. 2002. 14: 99-108.

Koh SD, Lee H, Ward SM, Sanders KM. The Mystery of the Interstitial cells of urinary bladder. Annu Rev Pharmacol Toxicol. 2018. 58: 603-623.

Komai AM, Brannmark C, Musovic S, Olofsson CS. PKA-independent cAMP stimulation of white adipocyte exocytosis and adipokine secretion: modulations by Ca^{2+} and ATP. J Physiol. 2014. 592: 5169-5186.

Komai AM, Musovic S, Peris E, Alrifaiy A, El Hachmane MF, Johansson M, Wernstedt Asterholm I, Olofsson CS. White adipocyte adiponectin exocytosis is stimulated via β3-adrenergic signaling and activation of Epac1: Catechol lamine resistance in obesity and type 2 diabetes. Diabetes. 2016. 65(11): 3301-3313.

Kono M, Nakamura Y, Ishiura Y, Komatsu K, Kontani H, Namiki M. Central muscarinic receptor subtypes regulating voiding in rats. J Urol. 2006. 175: 353-357.

Kooistra MR, Corada M, Dejana E, Bos JL. Epac1 regulates integrity of endothelial cell junctions through VE-cadherin. FEBS Lett. 2005. 579:4966–4972.

Kories C, Czyborra C, Fetscher C, Schneider T, Krege S, Michel M. Gender comparison of muscarinic receptor expression and function in rat and human urinary bladder: differential regulation of M2 and M3 receptor? Naunyn-Schim Arch Pharmacol. 2003. 367: 524-531.

Kruse R, Säve S, Persson K. Adenosine triphosphate induced P2Y2 receptor activation induces proinflammatory cytokine release in uroepithelial cells. J Urol. 2012. 188(6): 2419-25.

Kubo T, Fukuda K, Mikami A, Maeda A, Takahashi H, Mishina M, Haga T, Haga K, Ichiyama A, Kangawa K, Kojima A, Matsuo H, Hirose T, Numa S. Cloning, sequencing and expression of complementary DNS encoding the muscarinic acetylcholine receptor. Nature. 1986a. 323: 411-416.

Kukulski F, Lévesque SA, Lavoie EG, Lecka J, Bigonnesse F, Knowles AF, Robson SC, Kirley TL, Sévigny J. Comparative hydrolysis of P2 receptor agonist by NTPDases1,2,3 and 8. Purinergic Signal. 2005. 1:193-204.

Kullmann FA, Artim D, Beckel J, Barrick S, de Groat WC, Birder LA. Heterogeneity of muscarinic receptor-mediated Ca^{2+} responses in cultured urothelial cells from rat. Am J Physiol Renal Physiol. 2008. 294:F971–F981.

Kullmann FA, Limberg BJ, Artim DE, Shah M, Downs TR, Contract D, Wost J, Rosenbaum JS, de Groat WC. Effects of beta3-adrenergic receptor activation on rat urinary bladder hyperactivity induced by ovariectomy. J Pharmacol Exp Ther. 2009. 330: 704-717.

Kumar V, Chapple CC, Chess-Williams R. Characteristics of adenosine triphosphate release from porcine and human normal bladder. J Urol. 2004. 172: 744-747.

Kumar V, Chapple CR, Rosario D, Tophill PR, Chess-Williams. In vitro release of adenosine triphosphate from urothelium of human bladders with detrusor overactivity, both neurogenic and idiopathic. Eur Urol. 2010. 57: 1087-1092.

Kumar V, Templeman L, Chapple CR, Chess-Williams R. Recent developments in the management of detrusor overactivity. Curr Opin Urol. 2003. 13: 285-91.

Kunapuli SP, Dorsam RT, Kim S, Quinton TM. Platelet purinergic receptors. Curr Opin Pharmacol. 2003. 3: 175-80.

Kushida N, Fry CH. On the origin of spontaneous activity in the bladder. BJU Int. 2016. 117(6): 982-92.

Landers ES, Linton LM, Birren B, Nusbaum C, Zody MC, Baldwin J, Devon K, Doyle M, FitzHugh W et al. Initial sequencing and analysis of the human genome. Nature. 2001. 409: 860-921.

Lands AM, Arnold A, McAuliff JB, Luduena FP, Brown Jun TG. Differentiation of receptor system activated by sympatomimetic amines. Nature. 1967. 214: 597-8.

Laurenza A, Sutkowski E, Seamon KK. Forskolin: a specific stimulator of adenylate cyclase or a diterpene with multiple sites of action. Trends Pharmacol Sci. 1989. 10: 442-447.

Lawrence GW, Aoki KR, Dolly JO. Excitatory cholinergic and purinergic signaling in bladder are equally susceptible to botulinum neurotoxin a consistent with co-release of transmitters from efferent fibers. J Pharmacol Exp Ther. 2010. 334(3): 1080-6.

Lazarowski ER, Boucher RC, Harden TK. Mechanisms of release of nucleotides and integration of their actions as P2X-and P2Y-receptor activating molecules. Mol Pharmacol. 2003. 64: 785-95.

Le Novére N, Changeus JP. Molecular evolution of the nicotinic acetylcholine receptor: an example of multigene family in excitable cells. J Mol Evol. 1995. 40: 155-172.

Leblais V, Demolombe S, Vallette G, Langin D, Baró I, Escande D, Gauthier C. β_3-Adrenoceptor control the cystic fibrosis transmembrane conductance regulator

through a cAMP/Protein kinase A-independent pathway. J Biol Chem. 1999. 274: 6107-6113.

Lecci A, Giuliani S, Tramontana M, Santicioli P, Criscuoli M, Dion S, Maggi CA. Bladder distension and activation of the efferent function of sensory fibers: similarities with the effect of capsaicin. Br J Pharmacol. 1998. 124: 259-266.

Lee HY, Bardini M, Burnstock G. Distribution of P2X receptors in the urinary bladder and the ureter of the rat. J Urol. 2000. 163: 2002-7.

Lefkowitz RJ, Shenoy SK. Transduction of receptor signals by beta-arrestins. Science. 2005. 308: 512-517.

Lefkowitz RJ. G protein-coupled receptors. III. New roles for receptor kinases and beta-arrestins in receptor signaling and desensitization. J Biol Chem. 1998. 273: 18677-18680.

Lepor H. Pathophysiology, epidemiology, and natural history of benign prostatic hyperplasia. Rev Urol. 2004. (6):9.

Lévesque SA, Lavoie ÉG, Lecka J, Bigonness F, Sévigny J. Specificity of the ecto-ATPase inhibitor ARL67156 on human and mouse ectonucleotidases. Br J Pharmacol. 2007. 152: 141-150.

Levin RM, Haugaard N, O'Connor L, Buttyan R, Das A, Dixon JS, Gosling JA. Obstructive response of human bladder to BPH vs. rabbit bladder response to partial outlet obstruction: a direct comparison. Neurourol Urodyn. 2000. 19(5): 609-29.

Levin RM, Wein AJ. Distribution and function of adrenergic receptors in the urinary bladder of the rabbit. Molec Pharmacol. 1979. 16: 441-448.

Lewis SA, Diamond JM. Active sodium transport by mammalian urinary bladder. Nature. 1975. 253:747-8.

Lewis SA, Hanrahan JW. Apical and basolateral membrane ionic channels in rabbit urinary bladder epithelium. Pflugers Arch. 1985. 405 Suppl 1:S83-8.

Lewis SA, Lewis JR. Kinetics of urothelial ATP release. Am J Physiol Renal Physiol. 2006. 291: F332-F340.

Lewis SA. Everything you wanted to know about the bladder epithelium but were afraid to ask. Am J Physiol. 2000. 278, F867–F874.

Li J, O'Connor KL, Cheng X, Mei FC, Uchida T, Townsend CM Jr, Evers BM. Cyclic adenosine 5′-monophosphate-stimulated neurotensin secretion is mediated through Rap1 downstream of both Epac and protein kinase A signaling pathways. Mol Endocrinol. 2007. 21:159–171.

Li Y, Xue L. Mao Q, Yao L, Yuan J, Qin W, Zhao Y, Sun H, Liu F, Wang H. Expression and electrophysiological characteristics of P2X3 receptors in interstitial cells of Cajal in rats with partial bladder outlet obstruction. BJU Int. 2013. 111(5): 843-51.

Liang FX, Riedel I, Deng FM, Zhou G, Xu C, Wu XR, Kong XP, Moll R, Sun TT. Organization of uroplakin subunits: transmembrane topology, pair formation and plaque composition. J Biochem. 2001. 355:13–18.

Limberg BJ, Andersson KE, Kullmann AF, Burmer G, de Groat WC, Rosenbaum JS. β-Adrenergic receptor subtype expression in myocyte and non-myocyte cells in human female bladder. Cell Tissue Res. 2010. 342(2): 295-306.

Lips KS, Wunsch J, Zarghooni S, Bschleipfer T, Schukowski K, Weidner W, Wessler I, Schwantes U, Koepsell H, Kummer W. Acetylcholine and molecular components of its synthesis and release machinery in the urothelium. Eur Urol. 2007. 51(4): 1042-53.

Listerud M, Brussaard AB, Devay P, Colman DR, Role LW. Functional contribution of neuronal nicotinic subunits revealed by antisense oligonucleotides. Science. 1991. 254: 1518-1521.

Lodish H, Berk A, Lawrence Zipursky S, Matsudaira P, Baltimore D, Darnell J. Molecular Cell Biology. 4th edition. New York: WH Freeman. 2000.

Loffler M, Morote-Garcia JC, Eltzschig SA, Coe IR, Eltzschig HK. Physiological roles of vascular nucleoside transporters. Arterioscler Thromb Vasc Biol. 2007. 27: 1004-1013.

Losavio A, Muchnik S. Facilitation of spontaneous acetylcholine release induced by activation of cAMP in rat neuromuscular junctions. Life Sci. 2000. 66 (26): 2543-56.

Lu SH, Groat WC, Lin AT, Chen KK, Chang LS. Evaluation of purinergic mechanisms for the treatment of voiding dysfunction: a study in conscious spinal cord-injured rats. J Chin Med Assoc. 2007. 70: 439-444.

Lukas RJ, Changeux JP, Le Novére N, Albuquerque EX, Balfour DJ, Berg DK, Bertrand D, Chiappinelli VA, Clarke PB, Collins AC, Dani JA, Grady SR, Kellar KJ, Lindstrom JM, Marks MJ, Quik M, Taylor PW, Wonnacott S. International Union of Pharmacology XX. Current status of the nomenclature for nicotinic acetylcholine receptors and their subunits. Pharmacol Rev. 1999. 51, 397-401.

Lukas RJ. Diversity and patterns of regulation of nicotinic receptor subtypes. Ann N Y Acad Sc. 1995. 757: 153-168.

Lythgoe C, McVary KT. The use of PDE-5 inhibitors in the treatment of lower urinary tract symptoms due to benign prostatic hyperplasia. Curr Urol Rep. 2013. 14: 585-94.

Macarak EJ, Howard PS. The role of collagen in bladder filling. Adv Exp Med Biol. 1999. 462:215–223.

Magalhães-Cardoso MT, Pereira MF, Oliveira L, Ribeiro JÁ, Cunha RA, Correia-de-Sá P. Ecto-AMP deaminase blunts the ATP-derived adenosine A2A receptor facilitation of acetylcholine release at rat motor nerve endings. J Physiol. 2003. 549: 399-408.

Maggi CA, Meli A. Modulation by β-adrenoceptors of spontaneous contractions of rat urinary bladder. J Auton Pharmacol. 1982. 2: 255-261.

Maggi CA, Santicioli P, Geppetti P, Frilli S, Spillantini MG, Nediani C, Hunt SP, Meli A. Biochemical, anatomical and functional correlates of postnatal development of the capsaisin-sensitive innervation of the rat urinary bladder. Brain Res. 1988. 471: 183-190.

Maggi CA, Santicioli P, Geppetti P, Furio M, Frilli S, Conte B, Fanciullacci M, Giuliani S, Meli A. The contribution of capsaicin-sensitive innervation to activation of the spinal vesicovesical reflex in rats: relationship between substance P levels in the urinary bladder and the sensory-efferent function of capsaicin-sensitive sensory neurons. Brain Res. 1987. 415: 1-13.

Maggi CA, Santicioli P, Meli A. Pharmacological evidence for the existence of two components in the twitch response to field stimulation of detrusor strips from the rat urinary bladder. J Auton Pharmacol. 1985. 5: 221-229.

Mahony DT, Laferte RO, Blais DJ. Integral storage and voiding reflexes. Neurophysiologic concept of continence and micturition. Urol. 1977. 9: 95-106.

Maillet M, Robert SJ, Cacquevel M, Gastineau M, Vivien D, Bertoglio J, Zugaza JL, Fischmeister R, Lezoualc'h F. Crosstalk between Rap1 and Rac regulates secretion of sAPPalpha. Nat Cell Biol. 2003. 5:633–639.

Mandelzys A, Pié B, Deneris ES, Cooper E. The developmental increase in acetylcholine current densities on rat sympathetic neurons correlates with changes in nicotinic ACh receptor alpha-subunit gene expression and occurs independent of innervation. J Neurosci. 1994. 14: 2357-2364.

Mangera A, Apostolidis A, Andersson KE, Dasgupta P, Giannantoni A, Roehrborn C, Novara G, Chapple C. An update systematic review and statistical comparison of standardized mean outcomes for the use of botulinum toxin in the management of lower urinary tract disorders. Eur Urol. 2014. 65: 981-90.

Mansfield KJ, Liu L, Mitchelson FJ, Moore KH, Millard RJ, Burcher E. Muscarinic receptor subtypes in human bladder detrusor and mucosa, studied by radioligand binding and quantitative competitive RT-PCR: changes in aging. Br J Pharmacol. 2005. 144: 1089-1099.

Mao D, Yasuda RP, Fan H, Wolfe BB, Kellar KJ. Heterogeneity of nicotinic cholinergic receptors in rat superior cervical and nodose ganglia. Mol Pharmacol. 2006. 70: 1693-1699.

Marteau F, Le Poul E, Communi D, Communi D, Labouret C, Savi P, Boeynaems JM, Gonzalez NS. Pharmacological characterization of the human P2Y13 receptor. Mol Pharmacol. 2003. 64(1): 104-12.

Martin BF. Cell replacement and differentiation in transitional epithelium: a histological and autoradiographic study of the guinea-pig bladder and ureter. J Anat. 1972. 112:433-55.

Masunaga K, Chapple CR, McKay NG, Yoshida M, Sellers DJ. The β3-adrenoceptor mediates the inhibitory effects of β-adrenoceptor agonists via the urothelium in pig bladder dome. Neurourol Urodyn. 2010. 29: 1320-1325.

Matos S, Cordeiro JM, Coelho A, Ferreira S, Silva C, Igawa Y, Cruz F, Charrua A. Bladder pain induced by prolonged peripheral alpha 1a-adrenoceptor stimulation involves the enhancement of transient receptor potential vanilloid 1 activity and an increase of urothelial adenosine triphosphate release. Acta Physiol. 2016. 218: 265-275.

Matsui M, Griffin MT, Shehnaz D, Takedo MM, Ehlert FJ. Increased relaxant action of forskolin and isoproterenol against muscarinic agonist-induced contraction in smooth muscle from M2 receptor knockout mice. J Pharm. Exp. Therap. 2003. 305: 106-113.

Matsui M, Motomura D, Karasawa H, Fujikawa T, Jiang J, Komiya Y, Takahashi A, Taketo MM. Multiple functional defects in peripheral autonomic organs in mice lacking muscarinic acetylcholine receptor gene for the M3 subtype. Proc Nat Acad Sci. 2000. 97: 9579-9584.

Matsumoto Y, Miyazato M, Furuta A, Torimoto K, Hirao Y, Chancellor MB, Yoshimura N. Differential roles of M2 and M3 muscarinic receptor subtypes in modulation of bladder afferent activity in rats. Urology. 2010. 75(4): 862-867.

Matsumoto Y, Miyazato M, Yokoyama H, Kita M, Hirao Y, Chancellor MB, Yoshimura N. Role of M2 and M3 muscarinic acetylcholine receptor subtypes in activation of bladder afferent pathways in spinal cord injured rats. Urology. 2012. 79(5): 1184.e15-1184.e20.

Matsuoka I, Ohkubo S. ATP- and adenosine-mediated signaling in the central nervous system: adenosine receptor activation by ATP through rapid and localized generation of adenosine by ecto-nucleotidases. J Pharmacol Sci. 2004. 94(2): 95-9.

McCloskey KD. Bladder interstitial cells: an update review of current knowledge. Act Physiol. 2013. 207: 7-15.

McCloskey KD. Interstitial cells in the urinary bladder – Localization and function. Neuroutol Urodyn. 2010. 29: 82-87.

McHugh EM, McGee R. Direct anesthetic-like effects of forskolin on the nicotinic acetylcholine receptors of PC12 cells. J Biol Chem. 1986. 261: 3103-3106.

McLatchie LM, Fry CH. ATP release from freshly isolated guinea-pig bladder urothelial cells: a quantification and study of the mechanisms involved. BJU Int. 2014. 115 (6): 987-93.

McLatchie LM, Fry CH. ATP release from freshly isolated guinea-pig bladder urothelial cells: a quantification and study of the mechanisms involved. BJU Int. 2015. 115(6): 987-93.

Meek PD, Evang SD, Tadrous M, Roux-Lirange D, Triller DM, Gumustop B. Overactive bladder drugs and constipation: a meta-analysis of randomized, placebo-controlled trials. Dig Dis Sci. 2011. 56:7-18.

Merrill L, Gonzalez EJ, Girard BM, Vizzard MA. Receptors, channels, and signaling in the urothelial sensory system in the bladder. Nat Rev Urol. 2016. 13(4): 193-204.

Michel MC, Korstanje C. β3-Adrenoceptor agonists for overactive bladder syndrome: Role of translational pharmacology in a repositioning clinical drug development project. Pharmacol Ther. 2016. 159: 66-82.

Michel MC, Vrydag W. Alpha1-, alpha2- and beta-adrenoceptors in the urinary bladder, urethra and prostate. Br J Pharmacol. 2006. 147(Suppl 2):S88-119.

Mirone V, Imbimbo C, Longo N, Fusco F. The detrusor muscle: an innocent victim of bladder outlet obstruction. Eur Urol. 2007. 51: 57.

Miyamato T, Mochizuki T, Nakagomi H, Kira S, Watanabe M, Takayama Y, Suzuki Y, Koizumi S, Takeda M, Tominaga M. Functional role of Piezo1 in stretch-evoked Ca^{2+} influx and ATP release in urothelial cell cultures. J Biol Chem. 2014. 289(23): 16565-75.

Mochizuki T, Sokabe T, Araki I, Fujishita K, Shibasaki K, Uchida K, Naruse K, Koizumi S, Takeda M, Tominaga M. The TRPV4 cation channel mediates stretch-evoked Ca^{2+} influx and ATP release in primary urothelial cell cultures. J Biol Chem. 2009. 284(32): 21257-64.

Mochizuki T, Sokabe T, Araki I, Fujishita K, Shibasaki K, Uchida K, Naruse K, Koizumi S, Takeda M, Tominaga M. The TRPV4 cation channel mediates stretch-evoked Ca^{2+} influx and ATP release in primary urothelial cell cultures. J Biol Chem. 2009. 284: 21257-64.

Moore KH, Ray FR, Barden JA. Loss of purinergic P2X3 and P2X5 receptor innervation in human detrusor from adults with urge incontinence. J Neurosci. 2001. 21: 1-6.

Moro C, Chess-Williams R. Non-adrenergic, non-cholinergic, non-purinergic contractions of the urothelium/lamina propria of the pig bladder. Auton Autacoid Pharmacol. 2012. 32: 53.

Morrins JL, Gibbins IL. Co-transmission and neuromodulation. In: Burnstock G, Hoyle CHV (eds) Autonomic neuroeffector mechanisms. Harwood Academic Publishers. 1992: 33-119.

Morrison J, Birder L, Craggs M, et al. Incontinence. Abrams P, Cardozo L, Khoury S, Wein A, editors. Health Publications Ltd; Jersey: 2005. 363-422.

Morrison JFB. The activation of bladder wall afferent nerves. Exp Physiol. 1999. 84: 131-136.

Mukerji G, Yiangou Y, Grogono K, Underwood J, Agarwal SK, Khullar V, Anand P. Localization of M2 and M3 muscarinic receptors in human bladder disorders and their clinical correlations. J Urol. 2006. 176(1): 367-73.

Munoz A, Gangitano DA, Smith CP, Boone TB, Somogyi GT. Removal of urothelium affects bladder contractility and release of ATP but not release of NO in rat urinary bladder. BMC Urol. 2010. 24;10 :10.

Munoz A, Smith CP, Boone TB, Somogyi GT. Overactive and underactive bladder dysfunction is reflected by alterations in urothelial ATP and NO release. Neurochem Int. 2011. 58(3): 295-300.

Munoz A, Somogyi GT, Boone TB, Ford AP, Smith CP. Modulation of bladder afferent signals in normal and spinal cord-injured rats by purinergic P2X3 and P2X2/3 receptors. BJU Int. 2012. 110(800): E409-E414.

Murakami S, Chapple CR, Akino H, Sellers DJ, Chess-Williams R. The role of the urothelium in mediating bladder responses to isoprenaline. BJU Int. 2007. 99:669-673.

Murray JM, Bussiere DE. Targeting the purinome. Methods Mol Biol. 2009. 575: 47-92.

Mutafova-Yambolieva VN, Durnin L. The purinergic neurotransmitter revisited: a single substance or multiple players? Pharmacol Ther. 2014. 144: 162-91.

Nakagomi H, Yoshiyama M, Mochizuki T, Miyamoto T, Komatsu R, Imura Y, Morizawa Y, Hiasa M, Takaaki M, Kira S, Araki I, Fujishita K, Shibata K, Shigetomi E, Shinozaki R, Ichikawa R, Uneyama H, Iwatsuki K, Nomura M, de Groat WC, Moriyama Y, Takeda M, Koizumi S. Urothelial ATP exocytosis: regulation of bladder compliance in the urine storage phase. Sci Rep. 2016. 6: 29761.

Namasivayam S, Eardley I, Morrison JFB. Purinergic sensory neurotransmission in the urinary bladder: an in vitro study in the rat. BJU Int. 1999. 84: 854-860.

Negoro H, Lutz SE, Liou LS, Kanematsu A, Ogawa O, Scemes E, Suadicani SO. Pannexin 1 involvement in bladder dysfunction in a multiple sclerosis model. Scient Reports. 2013. 3: 2152.

Negoro H, Urban-Maldonado M, Liou LS, Spray DC, Thi MM, Suadicani SO. Pannexin 1 channels play essential roles in urothelial mechanotransduction and intercellular signaling. Plos One. 2014. 9(8): e106269.

Nicholls J, Hourani SM, Kitchen I. Characterization of P1-purinoceptors on rat duodenum and urinary bladder. Br J Pharmacol. 1992. 105: 639-642.

Nitti VW, Rosenberg S, Mitcheson DH, He W, Fakhoury A, Martin NE. Urodynamics and safety of the β3- adrenoceptor agonist mirabegron in males with lower urinary tract symptoms and bladder outlet obstruction. J Urol. 2013. 190: 1320-1327.

Nomiya M, Yamaguchi O. A quantitative analysis of mRNA expression of α1 and β-adrenoceptor subtypes and their functional roles in human normal and obstructed bladders. J Urol. 2003. 170: 649-653.

North RA. Molecular physiology of P2X receptors. Physiol Rev. 2002. 82: 1013-1067.

Novak I. ATP as a signaling molecule: the exocrine focus. News in Physiol Sci. 2003. 18(1): 12-17.

Novara G, Galfano A, Secco S, D'Elia C, Cavalleri S, Ficarra V, Artibani W. A systematic review and meta-analysis of randomized controlled trials with antimuscarinic drugs for overactive bladder. Eur Urol. 2008. 54: 740-63.

O´Reilly BA, Kosaka AH, Knight GF, Chang TK, Ford AP, Rymer JM, Popert R, Burnstock G, McMahon SB. P2X receptors and their role in female idiophatic detrusor instability. J Urol. 2002. 167: 157-164.

O'Reilly BA, Kosaka AH, Chang TK, Ford AP, Popert R, McMahon SB. A quantitative analysis of purinoceptor expression in the bladders of patients with symptomatic outlet obstruction. BJU Int. 2001a. 87: 617-622.

O'Reilly BA, Kosaka AH, Chang TK, Ford AP, Popert R, Rymer JM, McMahon SB. A quantitative analysis of purinoceptors expression in human fetal and adult bladders. J Urol. 2001b. 165(5): 1730-4.

Obrara K, Lepor H, Walden PD. Localization of $P2Y_1$ purinoceptors transcripts in the rat penis and urinary bladder. J Urol. 1998. 160: 587-591.

Ochodnicky P, Uvelius B, Anderson KE, Michel MC. Autonomic nervous control of the urinary bladder. Acta Physiol. 2013. 207-16.

Ockrim JL, Laniado ME, Patel A, Tubaro A, S St Clair Carter. A probability based system for combining simple office parameters as a predictor of bladder outflow obstruction. J Urol. 2001. 166(6): 2221-2225.

Oelke M, Höfner K, Jonas U, de la Rosette JJ, Ubbink DT, Wijkstra H. Diagnostic Accuracy of Noninvasive Tests to Evaluate Bladder Outlet Obstruction in Men: Detrusor Wall Thickness, Uroflowmetry, Postvoid Residual Urine, and Prostate Volume. Eur Urol. 2007. 52 (3): 827-835.

Ogoda M, Ito Y, Fuchihata Y, Onoue S, Yamada S. Characterization of muscarinic and P2X receptors in the urothelium and detrusor muscle of the rat bladder. J Pharmacol Sci. 2016. 131(1): 58-63.

Oike, M, Creed KE, Onoue H, Tanaka H, Ito Y. Increase in calcium in smooth muscle cells of the rabbit bladder induced by acetylcholine and ATP. J Auton Nerv Syst. 1998. 69:141-147.

Otsuka A, Kawasaki H, Matsumoto R, Shinbo H, Kurita Y, Iwashita T, Ozono S. Expression of β-adrenoceptor in the human urinary bladder urothelium. Naunyn Scmiedeberg's Arch Pharmacol. 2008. 377: 473-81.

Owen SJ, Massa HH, Rose'Meyer BB. Loss of adenosine A2B receptor mediated relaxant responses in the aged female rat bladder; effects of dietary phytoestrogens. Naunyn Schmiedebergs Arch Pharmacol. 2012. 385: 539-549.

Ozaki N, Shibasaki T, Kashima Y, Miki T, Takahashi K, Ueno H, Sunaga Y, Yano H, Matsuura Y, Iwanaga T, Takai Y, Seino S. cAMP-GEFII is a direct target of cAMP in regulated exocytosis. Nat Cell Biol. 2000. 2:805–811.

Ozawa H, Chancellor MB, Jung SY, Yokoyama T, Fraser MO, Yu Y, de Groat WC, Yoshimura N. Effect of intravesical nitric oxide therapy on cyclophosphamide-induced cystitis. 1999. J Urol, 162(6): 2211-2216.

Package insert. Myrbetriq (mirabegron). Norman, OK: Astellas Pharma Tecnhologies, Inc., June 2012.

Paes-De-Carvalho R. Adenosine as a signaling molecule in the retina: biochemical and developmental aspects. An Acad Bras Cienc. 2002. 74(3): 437-51.

Pakzad M, Ikeda Y, McCarthy C, Kitney DG, Jabr RI, Fry CH. Contractile effects and receptor analysis of adenosine-receptors in human detrusor muscle from stable and neuropathic bladders. Naunyn Schmiedebergs Arch Pharmacol. 2016. 389(8): 921-9.

Palea S, Artibani W, Ostardo E, Trist DG, Pietra C. Evidence for purinergic neurotransmission in human urinary bladder affected by interstitial cystitis. J Urol. 1993. 150(6): 2007-12.

Palea S, Corsi M, Pietra W, Artibani W, Calpista A, Gaviraghi G, Trist DG. ADP beta S induces contraction of the human isolated urinary bladder through a purinoceptor subtype different from P2X and P2Y. J Pharmacol Exp Ther. 1994. 269: 193-197.

Palmer RK, Boyer JL, Schacter JB, Nicolas RA, Harden TK. Agonist action of adenosine triphosphate at the human $P2Y_1$ receptor. Mol Pharmacol. 1998. 54: 1118-1123.

Pandita RK, Mizusawa H, Andersson KE. Intravesical oxyhemoflobin initiates bladder overactivity in conscious, normal rats. J Urol. 2000. 164: 545-550.

Parija SC, Raviprakash V, Mishra SK. Adenosine- and α,β.methylene ATP-induced differential inhibition of cholinergic and non-cholinergic responses in rat urinary bladder. Br J Pharmacol. 1991. 102: 396-400.

Park JM, Bloom DA, McGuire EJ. The guarding reflex revisited. Br J Urol. 1997. 80: 940-945.

Paukert M, Osteroth R, Geisler HS, Brändle U, Glowatzki E, Ruppersberg JP, Gründer S. Inflammatory mediator potentiat ATP-gated channels through the P2X(3) subunit. J Biol Chem. 2001. 276: 21077-21082.

Peralta EG, Ashkenazi A, Winslow JW, Ramachandran J, Capon DJ. Differential regulation of PI hydrolysis and adenylyl cyclase by muscarinic receptor subtypes. Nature. 1988. 334: 434-437.

Peralta EG, Ashkenazi A, Winslow JW, Smith DH, Ramachandran J, Capon DJ. Distinct primary structures, ligand-binding properties and tissue-specific expression or four human muscarinic acetylcholine. EMBO. 1987. 6: 3923-3929.

Persyn S, De Wachter S, Wyndaele JJ, Eastham J, Gillespie J. The actions of isoprenaline and mirabegron in the isolated whole rat and guinea pig bladder. Auton Neurosci. 2016a. 198: 19-27.

Persyn S, Eastham J, De Wachter S, Gillespie J. Adrenergic signaling elements in the bladder wall of the adult rat. Auton Neurosci. 2016b. 201: 40-48.

Petkov GV. Central role of the BK channel in urinary bladder smooth muscle physiology and pathophysiology. Am J Physiol Regul Integr Comp Physiol. 2014. 307(6): 571-84.

Picher M, Boucher RC. Human airway ecto-adenylate kinase. A mechanism to propagate ATP signaling on airway surfaces. J Biol Chem. 2003. 278: 11256-64.

Poth K, Nutter TJ, Cuevas J, Parker MJ, Adams DJ, Luetje CW. Heterogeneity of nicotinic receptors class and subunit mRNA expression among individual parasympathetic neurons from rat intracardiac ganglia. J Neurosci. 1997. 17: 586-596.

Prakasam HS, Herrington H, Roppolo JR, Jackson EK, Apodaca G. Modulation of bladder function by luminal adenosine turnover and A1 receptor activation. Am J Physiol Renal Physiol. 2012. 303: F279-F292.

Provophys et al., Human Physiology; Wikibooks contributors. 2006-2007. chapter 10.

Pullen AH, Tucker D, Martin JE. Morphological and morphometric characterization of Onuf's nucleus in the spinal cord in man. J Anat. 1997. 191: 201-213.

Qu HC, Zhang W, Liu YL, Wang PI. Association between polymorphism of β3-adrenoceptor gene and overactive bladder: a meta-analysis. Genet Mol Res. 2015. 14: 2495-501.

Ralevic V, Burnstock G. Receptors for purines and pyrimidines. Pharmacol Rev. 1998. 50: 413-492.

Ramer MS, Bradbury EJ, McMahon SB. Nerve growth factor induces P2X(3) Expression in sensory neurons. J Neurochem. 2001. 77: 864-875.

Rangarajan S, Enserink JM, Kuiperij HB, de Rooij J, Price LS, Schwede F, Bos JL. Cyclic AMP induces integrin-mediated cell adhesion through Epac and Rap1 upon stimulation of the beta 2-adrenergic receptor. J Cell Biol. 2003. 160:487–493.

Rapp DE, Lyon MB, Bales GT, Cook SP. A role for the P2X receptor in urinary tract physiology and the pathophysiology of urinary dysfunction. Eur Urol. 2005. 48: 303-308.

Ray FR, Moore KH, Hansen MA, Barden JA. Loss of purinergic P2X receptor innervation in human detrusor and subepithelium from adults with sensory urgency. Cell Tissue Res. 2003. 314: 351-359.

Reiter E, Lefkowitz RJ. GRKs and beta-arrestins: roles in receptor silencing, trafficking and signaling. Thends Endrocrinol Metab. 2006. 17: 159-165.

Reshkin SJ, Guerra L, Bagorda A, Debellis L, Cardone R, Li AH, Jacobson KA, Casavola V. Activation of A(3) adenosine receptor induces calcium entry and chloride secretion in A(6) cell. J Membr Biol. 2000. 178: 103-113.

Resta R, Yamashita Y, Thompson LF. Ecto-enzyme and signaling functions of lymphocyte CD73. Immunol Rev. 1998. 161: 95-109.

Roberts JA, Vial HR, Digby KC, Agboh KC, Wen H, Atterbury-Thomas A, Evans RJ. Molecular properties of P2X receptors. Pflugers Arch. 2006. 452: 486-500.

Robson SC, Sévigny J, Zimmermann H. The E-NTPDase family of ectonucleotidases: Structure function relationships and pathophysiological significance. Purinergic Signal. 2006. 2: 409-430.

Rockenbach L, Braganhol E, Dietrich F, Figueiró F, Pugliese M, Edelweiss MI, Morrone FB, Sévigny J, Battastini AM. NTPDase3 and ecto-5'-nucleotidase/CD73 are differentially expressed during mouse bladder cancer progression. Purinergic Signal. 2014. 10(3): 421-30.

Roehrborn CG, Abrams P, Royner ES, Kaplan Sam Herschom S, Guan Z. Efficacy and tolerability of tolterodine extended-release in men with overactive bladder and urgency urinary incontinence. BJU Int. 2006. 97: 1003-6.

Rong W, Spyer M, Burnstock G. Activation and sensitization of low and high threshold afferent fibers mediated by P2X receptors in the mouse urinary bladder. J Physiol. 2002. 541: 591-600.

RØtterud R, Nesland JM, Berner A, Fosså SD. Expression of the epidermal growth factor receptor family in normal and malignant urothelium. BJU Int. 2005. 95 (9): 1344-1350.

Rouget C, Rekik M, Camparo P, Botto H, Rischmann P, Lluel P, Palea S, Westfall TD. Modulation of nerve-evoked contractions by β3-adrenoceptor agonism in human and rat isolated urinary bladder. Pharmacol Res. 2014. 80: 14-20.

Rubinstein R, Shalev M, Nissenkorn I, Cohen S. Effect of exogenous adenosine and its monophosphate on the contractile response to acetylcholine in the human isolated detrusor muscle strips. J Auton Pharmacol. 1998. 18: 99-104.

Ruggieri MR Sr, Braverman AS, Pontari MA. Combined use of alpha-adrenergic and muscarinic antagonists for the treatment of voiding dysfunction. J Urol. 2005. 174: 1743-8.

Ruggieri MR. Mechanisms of disease: role of purinergic signaling in the pathophysiology of bladder dysfunction. Nature Clin Pract Urol. 2006. 3: 206-215.

Sadananda P, Shang F, Liu L, Mansfield KJ, Burcher E. Release of ATP from rat urinary bladder mucosa: role of acid, vanilloids and stretch. Br J Pharmacol. 2009. 158: 1655-1662.

Sakagami H, Aoki J, Natori Y, Nishikawa K, Kakehi Y, Natori Y, Arai H. Biochemical and molecular characterization of a novel choline-specific glycerophosphodiester phosphodiesterase belonging to the nucleotide pyrophosphatase/phosphodiesterase family. J Biol Chem. 2005. 280: 23084-23093.

Santoso AG, Sonarno IA, Arsad NA, Liang W. The role of the urothelium and ATP in mediating detrusor smooth muscle contractility. Urology. 2010. 76: 1267 (e7-e12).

Sargent PB. The diversity of neuronal nicotinic acetylcholine receptors. Ann Rev Neurosci. 1993.16:403-443.

Sassi Y, Ahles A, Truong DJ, Baqi Y, Lee SY, Husse B, Hulot JS, Foinquinos A, Thum T, Müller CE, Dendorfer A, Laggerbauer B, Engelhardt S. Cardiac myocyte-secreted cAMP exerts paracrine action via adenosine receptor activation. J Clin Invest. 2014. 124: 5385-5397.

Säve A, Persson K. Extracellular ATP and P2Y receptor activation induce a proinflammatory host response in the human urinary tract. Infec Immun. 2010. 78(8): 3609-3615.

Schachter JB, Harden TK. An examination of deoxyadenosine 5´(alpha-thio)triphosphate as a ligand to define P2Y receptors and its selectivity as a low potency partial agonist of the P2Y1 receptor. Br J Pharmacol. 1997. 121(2): 338-44.

Schmidt M, Bienek C, van Koppen CJ, Michel MC, Jacobs KH. Differential calcium signaling by M2 and M3 muscarinic acetylcholine receptors in a single cell type. Naunyn-Schm Arch Pharmacol. 1995. 352: 469-476.

Schulte G, Fredholm BB. Human adenosine A(1), A(2A), A(2B), and A(3) receptors expressed in Chinese hamster ovary cells all mediate the phosphorylation of extracellular-regulated kinase ½. Mol Pharmacol. 2000. 58(3): 477-82.

Schulte G, Fredholm BB. Signalling from adenosine receptors to mitogen-activated protein kinases. Cell Signal. 2003. 15: 813-827.

Schwiebert EM, Zsembery A. Extracellular ATP as a signaling molecule for epithelial cells. Bioch Biophysica Acta. 2003. 1615: 7-32. Review.

Seamon KB, Padgett W, Daty JW. Forskolin: unique diterpene activator of adenylate cyclase in membranes and in intact cells. Proc Natl Acad Sci. 1981. 78: 3363-3367.

Searl TJ, Dynda DI, Alanee SR, El-Zawahry AM, McVary KT, Silinsky EM. A1 adenosine receptor-mediated inhibition of parasympathetic neuromuscular transmission in human and murine urinary bladder. J Pharmacol Exp Ther. 2016. 356(1): 116-22.

Seguchi H, Nishimura J, Zhou Y, Niiro N, Kumazawa J, Kanaide H. Expression of β3-adrenoceptors in rat detrusor smooth muscle. Journal of Urology. 1998. 159: 2197-2201.

Seino S, Shibasaki T. PKA-dependent and PKA-independent pathways for cAMP-regulated exocytosis. Physiol Rev. 2005. 85:1303–1342.

Sellers DJ, Chess-Williams R. Muscarinic agonists and antagonists: effect on the urinary bladder. Hands Exp Pharmacol. 2012. 208: 375.

Sellers DJ, Yamanishi T, Chapple CR, Couldwell C, Yasuda K, Chess-Williams R. M3 muscarinic receptors but not M2 mediate contraction of the porcine detrusor muscle in vitro. J Auton Pharmacol. 2000. 20: 171-176.

Sengupta JN, Gebhart GF. Mechanosensitive properties of pelvic nerve afferent fibers innervating the urinary bladder of the rat. J Neurophysiol. 1994. 72: 2420-2430.

Shachter JB, Harden TK. An examination of deoxyadenosine 5'(α-thio)triphosphate as a ligand to define P2Y receptors and its selectivity as a low potency partial agonist of the $P2Y_1$ receptor. Br J Pharmacol, 1997. 121: 338-344.

Shafik A. Neuronal innervation of urethral and anal sphincters: surgical anatomy and clinical implications. Curr Opin Obstet Gynecol. 2000. 12: 387-398.

Shah AP, Mevcha A, Wilby D, Alatsatianos A, Hardman JC, Jacques S, Wilton JC. Continence and Micturition: An Anatomical Basis. Clinical Anatomy. 2014. 27: 1275-1283.

Shaikh G, Cronstein B. Signaling pathways involving adenosine A2A and A2B receptors in wound healing and fibrosis. Purinergic Signal. 2016. 12(2): 191-7.

Shaver SR. P2Y receptors: biological advances and therapeutic opportunities. Curr Opin Drug Discov Develp. 2001. 4(5): 665-670.

Silva-Ramos I, Silva I, Duarte-Araújo M, Lafuente-de-Carvalho J, Correia-de-Sá P. Loss of balance between excitatory P2X3 and inhibitory A2 receptors contribute to cholinergic hyperactivity in obstructed human bladder. Clin Pharmacol Toxicol. 2010. 107 (Suppl 1), 112-161.

Silva-Ramos M, Silva I, Faria M, Magalhães-Cardoso MT, Correia J, Ferreirinha F, Correia-de-Sá P. Impairment of ATP hydrolysis decreases adenosine A1 receptor tonus favoring cholinergic nerve hyperactivity in the obstructed human urinary bladder. Purinergic Signal. 2015a. 11:595-606.

Silva-Ramos M, Silva I, Faria M, Magalhães-Cardoso MT, Correia-de-Sá P. Urinary Bladder Disorders: Is adenosine friend or foe? In Adenosine Receptors: Pharmacology, Functions and Therapeutic Aspects. Ed. K. Warrick, nova Science Publishers, Inc (USA). 2015b.

Silva-Ramos M, Silva I, Oliveira JC, Correia-de-Sá P. Increased urinary adenosine triphosphate in patients with bladder outlet obstruction due to benign prostate hyperplasia. Prostate. 2016a. 76: 1353-1363.

Silva-Ramos M, Silva I, Ferreirinha F, Faria M, Correia-de-Sá P. Overactive bladder (OAB): is there a place for drugs targeting the purinergic cascade? In *Overactive Bladder (OAB): Prevalence, Risk Factors and Management*. Ed. Elvira Larson, Nova Science Publishers, Inc (USA). 2016b. Chapter 6, pp. 127-156. ISBN: 978-1-63485-033-9.

Silva-Ramos M, Silva I, Oliveira O, Ferreira S, Reis MJ, Oliveira JC, Correia-de-Sá P. Urinary ATP may be a dynamic biomarker of detrusor overactivity in women with overactive bladder syndrome. Plos One. 2013. 8(5): e64696.

Sjögren C, Andersson KE, Husted S, Mattiasson A, Moller-Madsen B. Atropine resistance of transmurally stimulated isolated human bladder muscle. J Urol. 1982. 128: 1368.

Smith CP, Vemulakonda VM, Kiss S, Boone TB, Somogyi GT. Enhanced ATP release from rat bladder urothelium during chronic bladder inflammation: Effect of botulinum toxin A. Neurochem Int. 2005. 47(4): 291-7.

Smith PP. Purinoceptors and bladder dysfunction. Curr Bladder Dysfunct Rep. 2011. 6: 85-92.

Socodato R, Brito R, Calaza KC, Paes-de-Carvalho R. Developmental regulation survival by adenosine in the in vitro and in vivo avian retina depends on a shift of signaling pathways leading to CREB phosphorylation or dephosphorylation. J Neurochem. 2011. 116(2): 227-39.

Sout CE, Costantin JL, Naus CC, Charles AC. Intercellular calcium signaling in astrocytes via ATP release through connexin hemichannels. J Biol Chem. 2002. 277(12): 10482-10488.

Speakman MJ, Brading AF, Gilpin CJ, Dixon JS, Gilpin SA, Gosling JA. Bladder outflow obstruction – a cause of denervation supersensitivity. J Urol. 1987. 138: 1461-1466.

Spector DA, Wade JB, Dillow R, Steplock DA, Weinman EJ. Expression, localization, and regulation of aquaporin-1 to -3 in rat urothelia. Am J Physiol Renal Physiol. 2002. 282:F1034-42.

Staskin DR, Chawla RK, Oefelein MG. Pharmacodynamics of overactive bladder drugs: shifting the curve. Curr Bladder Dysfunct Rep. 2011. 6: 51-63.

Stefan C, Jansen S, Bollen M. Modulation of purinergic signaling by NPP-type ectophosphodiesterases. Purnergic Signa. 2006. 2: 361-370.

Stefan C, Jansen S, Bollen M. NPP-type ectophosphodiesterases: unity in diversity. Trends Biochem Sci. 2005. 30: 542-550.

Stehle JH, Rivkees SA, Lee JJ, Weaver DR, Deeds JD, Reppert SM. Molecular cloning and expression of the cDNA for a novel A2-adenosine receptor subtype. Mol Endocrinol. 1992. 6: 384-393.

Stella J, Bavaresco L, Braganhol E, Rockenbach L, Farias PF, Wink MR, Azambuja AA, Barrios CH, Morrone FB. Differential ectonucleotidase expression in human bladder cancer cell lines. Urol Oncol. 2010. 28(3): 260-7.

Stewart WF, Van Rooyen JB, Cundiff GW, Abrams P, Herzog AR, Corey R, Hunt TL, Wein AJ. Prevalence and burden of overactive bladder in the United States. World J Urol. 2003. 20: 327-36.

Strosberg AD. Structure, function, and regulation of adrenergic receptors. Protein Sci. 1993. 2(8): 1198-209.

Sugaya K, Mori S, Tsuchida S. Efferent and primary afferent neural pathways innervating the lower urinary tract of the cat. Part I. Efferent neural pathways. Nippon Hinyokika Gakkai Zasshi (Jpn J. Urol). 1988a. 79: 868-879.

Sugaya K, Nishijima S, Miyazato M, Ogawa Y. Central nervous control of micturition and urine storage. J Smooth Muscle Res. 2005. 41 (3): 117-132.

Suh YS, Ko KJ, Kim TH, Lee HS, Sung HH, Cho WJ, Lee M, Lee KS. Potential biomarkers for diagnosis of overactive bladder patients: urinary nerve growth factor, prostaglandin E2, and adenosine triphosphate. Int Neurourol J. 2017. 21(3): 171-177.

Sui G, Fry B, Montgomery M, Roberts M, Wu R, Wu C. Purinergic and muscarinic modulation of ATP release from the urothelium and its paracrine actions. Am J Physiol Renal Physiol. 2014. (306): F286-F298.

Sui GP, Rothery S, Dupont E, Fry CH, Severs NJ. Gap junctions and connexin expression in human suburothelial interstitial cells. BJU Int. 2002. 90: 118-129.

Sui GP, Wu C, Fry CH. Characterization of the purinergic receptor subtype on guinea-pig suburothelial myofibroblasts. BJU Int. 2006. 97(6): 1327-31.

Sun TT. Altered phenotype of cultured urothelial and other stratified epithelial cells: implications for wound healing. Am J Physiol Renal Physiol. 2006. 291:F9–F21.

Sun Y, Chai TC. Augmented extracellular ATP signaling in bladder urothelial cells from patients with interstitial cystitis. Am J Physiol Cell Physiol. 2006. 290: C27.

Sun Y, MaLossi J, Jacobs SC, Chai TC. Effect of doxazosin on stretch-activated adenosine triphosphate release in bladder urothelial cells from patients with benign prostatic hyperplasia. Urology. 2002. 60(2): 351-6.

Surprenant A, North RA. Signaling at purinergic P2X receptors. Annu Rev Physiol. 2009. 71: 333-359.

SvalØ J, Nordling J, Bouchelouche K, Andersson KE, Korstanje C, Bouchelouche P. The novel β_3-adrenoceptor agonist mirabegron reduces carbachol-induced contractile activity in detrusor tissue from patients with bladder outflow obstruction with or without detrusor overactivity. Eur J Pharmacol. 2013. 699 (1-3):101-105.

Svennersten K, Hallén-Grufman K, de Verdier PJ, Wiklund NP, Poljakovic M. Localization of P2X receptor subtypes 2, 3 and 7 in human urinary bladder. BMC Urol. 2015. 8: 15-81.

Takasu T, Ukai M, Sato S, Matsui T, Nagase I, Maruyama T, Sasamata M, Miyata K, Uchida H, Yamaguchi O. Effect of (R)-2-(2-aminothiazol-4-yl)-4'-{2-[(2-hydroxy-2-phenylethyl)amino]ethyl} acetanilide (YM178), a novel selective β3-adrenoceptor agonist, on bladder function. J Pharmacol Exp Ther. 2007. 321: 642-647.

Takeda H, Yamazaki Y, Akahane M, Igawa Y, Ajisawa Y, Nishizawa O. Role of β3-adrenoceptor in urine storage in the rat: comparison between the selective β3-adrenoceptor agonist, CL316,243, and various smooth muscle relaxants. J Pharmacol Exp Ther. 2000. 293: 939-345.

Takeda M, Obara K, Mizusawa T, Tomita Y, Arai K, Tsutsui T, Hatano A, Takahashi K, Nomura S. Evidence for β3-adrenoceptor subtypes in relaxation of the human urinary bladder detrusor: analysis by molecular biological and pharmacological methods. J Pharmacol Exp Ther. 1999. 288: 1367-1373.

Takezawa K, Kondo M, Kiuchi H, Ueda N, Soda T, Fukuhara S, Takao T, Miyagawa Y, Tsujimura A, Matsumoto-Miyai K, Ishida Y, Negoro H, Ogawa O, Nonomura N, Shimada S. Authentic role of ATP signaling in micturition reflex. Sci Repor. 2016. 6: 19585.

Tempest HV, Dixon AK, Turner WH, Elneil S, Sellers LA, Ferguson DR. P2X and P2X receptor expression in human bladder urothelium and changes in interstitial cystitis. BJU Int. 2004. 93(9): 1344-8.

Thiagamoorthy G, Giarenis I, Cardozo L. Early investigational β3 adrenoceptor agonists for the management of the overactive bladder syndrome. Expert Opin Investig Drugs. 2015. 24: 1299-1306.

Timóteo MA, Carneiro I, Silva I, Noronha-Matos JB, Ferreirinha F, Silva-Ramos M, Correia-de-Sá P. ATP released via pannexin-1 hemichannels mediates bladder overactivity triggered by urothelial P2Y6 receptors. Biochem Pharmacol. 2014. 87(2): 372-379.

Togashi H, Emala CW, Hall IP, Hirshman CA. Carbachol induced actin reorganization involves Gi activation of Rho in human airway smooth muscle. Am J Physiol. 1998. 274: L803-L809.

Tyagi P, Thomas CA, Yoshimura N, Chancellor MB. Investigations into the presence of functional β1, β2 and β3-adrenoceptors in urothelium and detrusor of human bladder. Int Braz J Urol. 2009. 35(1): 76-83.

Tyagi S, Tyagi P, Van-le S, Yoshimura N, Chancellor MB, de Miguel F. Qualitative and quantitative expression profile of muscarinic receptors in human urothelium and detrusor. J Urol. 2006. 176(4 Pt 1): 1673-8.

Uchida H, Shishido K, Nomiya M, Yamaguchi O. Involvement of cyclic AMP-dependent and –independent mechanisms in the relaxation of rat detrusor muscle via β-adrenoceptors. Eur J Pharmacol. 2005. 518: 195-202.

Uchiyama T, Chess-Williams R. Muscarinic receptor subtypes of the bladder and gastrointestinal tract. J Smooth Muscle Res. 2004. 40 (6): 237-247.

Uhlén M, Fagerberg L, Hallström BM, Lindskog C, Oksvold P, Mardinoglu A, Sivertsson Å, Kampf C, Sjöstedt E, Asplund A, Olsson I, Edlund K, Lundberg E, Navani S, Szigyarto CA, Odeberg J, Djureinovic D, Takanen JO, Hober S, Alm T, Edqvist PH, Berling H, Tegel H, Mulder J, Rockberg J, Nilsson P, Schwenk JM, Hamsten M, von Feilitzen K, Forsberg M, Persson L, Johansson F, Zwahlen M, von Heijne G, Nielsen J, Pontén F. Proteomics. Tissue-based map of the human proteome. Science. 2015. 347: 126419.

Valera S, Hussy N, Evans RJ, Adami N, North RA, Surprenant A, Buell G. A new class of ligand-gated ion channel defined by P2X receptor for extracellular ATP. Nature. 1994. 371: 516-519.

Van Aubel RA, Smeets PH, Peters JG, Bindels RJ, Russel FG. The MRP4/ABCC4 gene encodes a novel apical organic anion transporter in human

kidney proximal tubules: putative efflux pump for urinary cAMP and cGMP. J Am Soc Nephrol. 2002. 13: 595-603.

Van de Graaff, Ward Rhees. Human anatomy and Physiology, Editor by Wilhelm PB, McGraw-Hill companies. 2001.

Vander et al.: Human Physiology: The Mechanism of Body Function, Eighth Edition; The McGraw−Hill Companies, 2001. Chapter 16.

Vesela R, Aronsson P, Tobin G. Functional and morphological examination of P1A1 purinoceptors in the normal and inflamed urinary bladder of the rat. Auton Neurosci. 2011. 159: 26-31.

Vial C, Evans RJ. P2X receptor expression in mouse urinary bladder and the requirement of P2X(1) receptors for functional P2X receptor responses in the mouse urinary bladder smooth muscle. Br J Pharmacol. 2000. 131: 1489-1495.

Vieira C, Magalhães-Cardoso MT, Ferreirinha F, Silva I, Dias AS, Pelletier J, Sévigny J, Correia-de-Sá P. Feed-forward inhibition of CD73 and upregulation of adenosine deaminase contribute to the loss of adenosine neuromodulation in postinflammatory ileitis. Mediators Inflamm. 2014. 2014:254640.

Virgino C, Robertson G, Surprenant A, North RA. Trinitrophenyl-substituted nucleotides are potent antagonists selective for P2X1, P2X3 and heteromeric P2X2/3. Mol Pharmacol. 1998. 53: 969-973.

Vlaskovska M, Kasakov L, Rong W, Bodin P, Bardini M, Cockayne DA, Ford AP, Burnstock G. P2X3 knockout mice reveal a major sensory role for urothelially released ATP. J Neurosc. 2001. 21: 5670-5677.

Vollmayer P, Clair T, Goding JW, Sano K, Servos J, Zimmermann H. Hydrolysis of diadenosine polyphosphates by nucleotide pyrophosphatases/ phosphodiesterases. Eur J Biochem. 2003. 270: 297-8.

Volonté C, D'Ambrosi N. Membrane compartments and purinergic signaling: the purinome, a complex interplay among ligand, degrading enzymes, receptors and transporters. J FEBS. 2009. 276: 318-329.

Vorhoff T, Zimmermann H, Pelletier J, Sévigny J, Braun N. Cloning and characterization of the ecto-nucleotidase NTPDase3 from rat brain: Predicted secondary structure and relation to other members of the E-NTPDase family and actin. Purinergic Signal. 2005. 1: 259-270.

Waldo GL, Corbitt J, Boyer JL, Ravi G, Kim HS, Ji XD, Lacy J, Jacobson KA, Harden TK. Quantitation of the P2Y(1) receptor with a high affinity radiolabeled antagonist. Mol Pharmacol. 2002. 62(5): 1249-57.

Waldo GL, Harden TK. Agonist binding and Gq-stimulating activities of the purified human P2Y1 receptor. Mol Pharmacol. 2004. 65: 426-436.

Walker B. Renewal of cell populations in the female mouse. Am J Anat. 1960. 107, 95–105.

Wall MJ, Wigmore G, Lopatář J, Frenguelli BG, Dale N. The novel NTPDase inhibitor sodium polyoxotungstate (POM-1) inhibits ATP breakdown but also blocks central synaptic transmission, an action independent of NTPDase inhibition. Neuropharm. 2008. 55: 1251-1258.

Wallukat G. The β-adrenergic receptors. Herz. 2002. 27(7): 683-690.

Wang EC, Lee J, Ruiz WG, Balestreire EM, von Bodungen M, Barrick S, Cockayne DA, Birder LA, Apodaca G. ATP and purinergic receptor-dependent membrane traffic in bladder umbrella cells. J Clin Invest. 2005. 115(9): 2412-2422.

Wang EC, Lee JM, Johnson JP, Kleyman TR, Bridges R, Apodaca G. Hydrostatic pressure regulated ion transport in bladder uroepithelium. Am J Physiol Renal Physiol. 2003. 285:F651-63.

Wang EC, Lee JM, Ruiz WG, Balestreire EM, von Bodungen M, Barrick S, Cockayne DA, Birder LA, Apodaca G. ATP and purinergic receptor dependent membrane traffic in bladder umbrella cells. J Clin Invest. 2005. 115(9): 2412-2422.

Wang L, Jacobson SE, Bengtsson A, Erlinge D. P2 receptor mRNA expression profiles in human lymphocytes, monocytes and $CD34^+$ stem and progenitor cells. BMC Immunol. 2004. 5:16.

Wang P, Liu Z, Chen H, Ye N, Cheng X, Zhou J. Exchange proteins directly activated by cAMP (EPACs): Emerging therapeutic targets. Bioorg Med Chem Lett. 2017. 27(8): 1633-39.

Wang P, Luthin GR, Ruggieri MR. Muscarinic acetylcholine receptor subtypes mediating urinary bladder contractility and coupling to GTP binding proteins. J Pharmavol Exp Ther. 1995. 273: 959-966.

Wegener JW, Schulla V, Lee TS, Koller A, Feil S, Feil R, Kleppisch T, Klugbauer N, Moosmang S, Welling A, Hofmann F. An essential role of Cav1.2 L-type calcium channel for urinary bladder function. FASEB J. 2004. 18(10): 1159-61.

Wessler I, Kilbinger A, Bittinger F, Unger R, Kirkpatrick CJ. The non-neuronal cholinergic system in humans: Expression, function and pathophysiology. Life Sci. 2003. 72 (18-19): 2055-2061.

Wettstein MS, Buser L, Hermanns T, Roudnicky F, Eberli D, Baumeister P, Sulser T, Wild P, Poyet C. CD73 predicts favorable prognosis in patients with nonmuscle-invasive urothelial bladder cancer. Dis Markers. 2015. 785461.

Winder M, Tobin G, Zupančič D, Romih R. Signaling molecules in the urothelium. Biomed Rest Int. 2014. 297295.

Wiseman OJ, Brady CM, Hussain IF, Dasgupta P, Watt H, Fowler CJ, Landon DN. The ultrastructure of bladder lamina propria nerves in healthy subjects and patients with detrusor hyperreflexia. J Urol. 2002. 168:2040–2045.

Wood MW, Breitschwerdt EB, Nordone SK, Linder KE, Gookin JL. Uropathogenic E. coli promote a paracellular urothelial barrier defect characterized by altered tight junction integrity, epithelial cell sloughing and cytokine release. J Comp Pathol. 2012. 147:11–19.

Woods M, Carson N, Norton NW, Sheldon JH, Argentieri TM. Efficacy of the beta3-adrenergic receptor agonist CL316243 on experimental bladder hyperreflexia and detrusor instability in the rat. J Urol. 2001. 166(3): 1142-7.

Wróbel A, Rechberger T. The influence of Rho-kinase inhibition on acetic acid-induced detrusor overactivity. Neurourol Urodyn. 2015. 36(2): 263-270.

Wu C, Gui GP, Fry CH. Intracellular Ca^{2+} regulation and electrophysiological properties of bladder urothelium subjected to stretch and exogenous agonists. Cell Calc. 2011. 49: 395-399.

Wu X, Kong X, Pellicer A, Kreibich G, Sun T-T. Uroplakins in urothelial biology, function, and disease. Kidney International. 2009. 75(11):1153–1165.

Wu Y, Shi C, Deng J, Zhang X, Song B, Li L. Expression and function of muscarinic subtype receptors in bladder interstitial cells of cajal in rats. Cellular and Molecular Urol. 2014. 11(03): 1642-1647.

Wuest M, Eichhorn B, Grimm MO, Wirth MP, Ravens U, Kaumann AJ. Catecholamines relax detrusor through beta 2-adrenoceptors in mouse and beta 3-adrenoceptors in man. J Pharmacol Exp Ther. 2009. 328: 213-222.

Xin W, Li N, Fernandes VS, Petkov GV. Constitutively active PKA regulates neuronal acetylcholine release and contractility of guinea pig urinary bladder smooth muscle. Am J Physiol Renal Physiol. 2016. 310(11): 1377-84.

Xu L, Gebhart GF. Characterization of mouse lumbar splanchnic nerve and pelvic urinary bladder mechanosensory afferents. J Neurophysio. 2008. 99: 244-253.

Xu W, Gelber S, Orr-Urtreger A, Armstrong D, Lewis RA, Ou C-N, Patrick J, Role L, De Biasi M, Beaudet A. Megacystis, mydriasis, and ion channel defect in mice lacking the α3 neuronal nicotinic acetylcholine receptor. Proc Natl Acad Sci. 1999a. 96: 5746-5751.

Xu W, Orr-Urtreger A, Nigro F, Gelber S, Sutcliffe CB, Armstrong D, Patrick JW, Role LW, Beaudet AL, De Biasi M. Multiorgan autonomic dysfunction in mice lacking the β2 and the β4 subunits of neuronal nicotinic acetylcholine receptors. J Neurosc. 1999b. 19: 9298-9305.

Yamaguchi O, Chapple CR. Beta3-adrenoceptors in urinary bladder. Neurourol Urodyn. 2007. 26: 752-756.

Yamaguchi O. Beta3-adrenoceptors in human detrusor muscle. Urology. 2002. 59: S25-9.

Yamaguchi O. Latest treatment for lower urinary tract dysfunction: therapeutic agents and mechanism of action. Int J Urol. 2013. 20: 28-39.

Yamamichi F, Shigemura K, Behnsawy HM, Yamashita M, Shirakawa T, Fujisawa M. Beta-3 adrenergic receptors could be significant factor for overactive bladder-related symptoms. Int J Clin Pathol. 2015. 8(9): 11863-11870.

Yamanishi T, Chapple CR, Yasuda K, Chess-Williams R. The role of M2-muscarinic receptors in mediating contraction of the pig urinary bladder in vitro. Br J Pharmacol. 2000. 131: 1482-1488.7

Yamanishi T, Kaga K, Fuse M, Shibata C, Kamai T, Uchiyama T. The role of muscarinic receptor subtypes on carbachol-induced contraction of normal human detrusor and overactive detrusor associated with benign prostatic hyperplasia. J Pharmacol Sci. 2015. 128: 65-70.

Yamazaki Y, Takeda H, Akahane M, Igawa Y, Nishizawa O, Ajisawa Y. Species differences in the distribution of β-adrenoceptor subtypes in bladder smooth muscle. Br J Pharmacol. 1998. 124: 593-599.

Yang SJ, An JY, Shim JO, Park CH, Huh IH, Sohn UD. The mechanism of contraction by 2-chliriadenosine in cat detrusor muscle cells. J Urol. 2000. 163: 652-658.

Yegutkin GG, Henttinen T, Jalkanen S. Extracellular ATP formation on vascular endothelial cells is mediated by ecto-nucleotide kinase activities via phosphotransfer reactions. FASEB. 2001. 15: 251-60.

Yokokawa R, Akino H, Ito H, Zha X, Yokoyama O. Nerve growth factor release from the urothelium increases via activation of bladder C-fibers in rats with cerebral infarction. Neurourol Urodyn. 2017. 36(6): 1448-1455.

Yoshida M, Homm Y, Inadome A, Yono M, Seshita H, Miyamoto Y, Murakami S, Kawabe K, Ueda S. Age-related changes in cholinergic and purinergic neurotransmission in human isolated bladder smooth muscles. Exp Gerontol. 2001. 36(1):99-109.

Yoshida M, Masunaga K, Nagata T, Yono M, Homma Y. The forefront for novel therapeutic agent based on the pathophysiology of lower urinary tract dysfunction: pathophysiology and pharmacotherapy of overactive bladder. J Pharmacol Sci. 2010. 112(2): 128-134.

Yoshida M, Miyamae K, Iwashita H, Otani M, Inadome A. Management of detrusor dysfunction in the elderly: changes in acetylcholine and adenosine triphosphate release during aging. Urology. 2004. 63 (3 Suppl 1): 17-23.

Yoshimura N, Chancellor MB. Neurophysiology of lower urinary. Rev Urol. 2003. 5(8): 3-10.

Yoshimura N, de Groat WC. Neural control of the lower urinary tract. Int J Urol. 1997. 4: 111-125.

Yoshimura N, Seki S, de Groat WC. Nitric oxide modulates Ca^{2+} channels in dorsal root ganglion neurons innervating rat urinary bladder. J Neurophysiol. 2001. 86(1): 304-311.

Yu W, Robson SC, Hill WG. Expression and distribution of ectonucleotidases in mouse urinary bladder. Plos One. 2011. 6(4):e18704.

Yu W, Sun X, Robson SC, Hill WG. ADP-induced bladder contractility is mediated by $P2Y_{12}$ receptor and temporally regulated by ectonucleotidases and adenosine signaling. FASEB. 2014. 28(12): 5288-98.

Yu W, Sun X, Robson SC, Hill WG. Extracellular UDP enhances P2X-mediated bladder smooth muscle contractility via $P2Y_6$ activation of the phospholipase C / inositol triphosphate pathway. FASEB. 2013. 27(5): 1895-903.

Yu W, Zacharia LC, Jackson EK, Apodaca G. Adenosine receptor expression and function in bladder uroepithelium. Am J Physiol Cell Physiol. 2006. 291(2): C254-C265.

Yu Y, Daugherty SL, de Groat WC. Effects of nicotinic receptor agonists on bladder afferent nerve activity in an in vitro bladder-pelvic nerve preparation. Brain Res. 2016. 1637: 91-101.

Zarghooni S, Wunsch J, Bodenbenner M, Brüggmann D, Grando SA, Schwantes U, Wess J, Kummer W, Lips KS. Expression of muscarinic and nicotinic acetylcholine receptors in the mouse urothelium. Life Sciences. 2007. 80: 2308-2313.

Zhang FL, Luo L, Gustafson E, Palmer K, Qiao X, Fan X, Yang S, Laz TM, Bayne M, Monsma F JR. $P2Y_{13}$: identification and characterization of a novel Gαi-coupled ADP receptor from human and mouse. J Pharmacol Exp Ther. 2002. 301: 705-713.

Zhang YJ, Bai Q. Urinary prostaglandins E2 correlates to overactive bladder symptoms in patients with benign prostatic hyperplasia. Zhonghua Nan Ke Xue. 2014. 20(3): 244-8.

Zhu Y, Chen H, Boulton S, Mei F, Ye N, Melacini G, Zhou J, Cheng X. Biochemical and Pharmacological Characterization of ESI-09 Based EPAC inhibitors: Defining the ESI-09 “Therapeutic Window”. Scientific Report. 2015. 5: 9344.

Zimmermann H, Braun N, Kegel B, Heine P. New insights into molecular structure and function of ectonucleotidases in the nervous system. Neurochem Int. 1998. 32: 421-5.

Zimmermann H. 5’-nucleotidase-molecular structure and functional aspects. Biochem J. 1992. 285: 385-365.

Zimmermann H. Ectonucleotidases: some recent developments and a note on nomenclature. Drug Dev Res. 2001. 52: 44-56.

Zimmermann H. Extracellular metabolism of ATP and other nucleotides. Nauyn-Schm Arch Pharmacol. 2000. 362: 299-309.