European Journal of Pharmacology 758 (2015) 115–122

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Pulmonary, gastrointestinal and urogenital pharmacology

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

Gianluigi D'Agostino [a,*], Anna Maria Condino [a], Paolo Calvi [b]

[a] Department of Drug Science, Experimental Pharmacology Unit, University of Pavia, Italy
[b] Urological Unit, Santa Rita Clinic Hospital, Vercelli, Italy

ARTICLE INFO

Article history:
Received 13 May 2014
Received in revised form
24 March 2015
Accepted 25 March 2015
Available online 8 April 2015

Keywords:
Acetylcholine release
β_3-Adrenoreceptors
Human detrusor
Mirabegron
Overactive bladder
Pre-junctional receptors

ABSTRACT

Bladder overactivity (OAB) is a multifactorial bladder disorder that requires therapeutics superior to the current pharmacological treatment with muscarinic antagonists. β_3-adrenoceptor (β_3-ADR) agonists represent a novel promising approach that differently addresses the parasympathetic pathway, but the clinical efficacy of these drugs has not been fully elucidated to date. Therefore, we aimed to study the pharmacological mechanisms activated by β_3-ADR agonists at muscular and neural sites in the isolated human bladder. Detrusor smooth muscle strips obtained from male patients undergoing total cystectomy were labelled with tritiated choline and stimulated with electrical field stimulation (EFS). EFS produced smooth muscle contraction and simultaneous acetylcholine ([^{3}H]-ACh) release, which mostly reflects the neural origin of acetylcholine. Isoprenaline (INA), BRL37344 and mirabegron inhibited the EFS-evoked contraction and [^{3}H]-ACh release in a concentration-dependent manner, yielding concentration–response curves (CRCs) that were shifted to the right by the selective β_3-ADR antagonists L-748,337 and SR59230A. Based on the agonist potency estimates (pEC_{50}) and apparent affinities (pK_b) of antagonists evaluated from the CRCs of agonists, our data confirm the occurrence of β_3-ADRs at muscle sites. Moreover, our data are consistent with the presence of inhibitory β_3-ADRs that are functionally expressed at the neural site. Taken together, these findings elucidate the mechanisms activated by β_3-ADR agonists because neural β_3-ADRs participate in the inhibition of detrusor motor drive by reducing the amount of acetylcholine involved in the cholinergic pathway.

1. Introduction

Overactive bladder syndrome (OAB) is a symptomatic condition characterised by urgency, with or without urgency incontinence that is usually more frequent during the daytime (Abrams et al., 2002). OAB is currently pharmacologically treated with muscarinic antagonists, which are drugs with a challenging profile and marginal efficacy (Muhlstein and Deval, 2008). Thus, this suboptimal management calls for the development of superior therapeutics *via* the discovery of novel receptor targets that differently address the parasympathetic pathway. To this end, recent studies have focused on sympathetic neurotransmission, especially the β-adrenergic receptors (β-ADRs), which are currently classified into three subtypes (β_1, β_2 and β_3) (Bylund et al., 1994; Strosberg and Pietri-Rouxel, 1996). Their expression and relative abundance are documented in the urinary bladder of mammals (Ursino et al., 2009), but interestingly, the stimulation of the predominant β_3-subtype (Nomiya and Yamaguchi, 2003; Tyagi et al., 2009) in response to sympathetic nerve activity in the human bladder seems to represent the most relevant mechanism to determine bladder capacity without influencing bladder contraction (Yamaguchi, 2002; Yamanishi et al., 2006; Yamaguchi and Chapple, 2007). This evidence pointed toward the clinical utility of β_3-ADRs agonists, which metamorphosed from experimental tools into efficacious drugs in voiding disorders. However, significant improvements in key symptoms in OAB patients (Yoshimura et al., 2008), as well as the reduction of phasic activity during the filling phase reported in clinical trials (Andersson, 2013), point towards a comprehensive picture of the effectiveness of β_3-ADR agonists. Because the micturition cycle is controlled by a complex interplay of neural pathways, the functional significance of β_3-ADRs conceivably does not merely reside at a muscle site but may derive from the activation of additional inhibitory mechanisms that target both efferent and afferent neurons. In conditions of impairment, they may prove fundamental in generating voiding disorders (Ochodnicky et al., 2013), an hypothesis corroborated by recent findings in the detrusor muscles of animals (Gillespie et al., 2003; Masunaga et al.,

* Corresponding author. Tel.: +39 0382987398; fax: +39 0382987405.
E-mail address: dagg@unipv.it (G. D'Agostino).

http://dx.doi.org/10.1016/j.ejphar.2015.03.074
0014-2999/

2010) and humans (Biers et al., 2006; Afeli et al., 2013; Rouget et al., 2014), where significant inhibitions in the cholinergic and purinergic component of the neurogenic response were ascribed to neural β_3-ADRs (Afeli et al., 2013; Rouget et al., 2014).

However, the supposition that β_3-ADRs play an additional putative role in smooth muscle relaxation is based on indirect methods of measuring cholinergic neurotransmission, which are not predictive of changes in ACh release per se. In this study of human detrusor strips, the [^{3}H]-ACh release and contractile response were measured simultaneously using a validated protocol that ascertains the direct involvement of pre-junctional receptors in the modulation of neurotransmitter release from parasympathetic terminals (D'Agostino et al., 2006). The simultaneous assessment of both pre- and post-junctional effects evoked by EFS demonstrated that both muscle and neural mechanisms are involved in the efficacy of β_3-ADR agonists, such as mirabegron, a drug recently approved for OAB treatment (Andersson, 2013).

2. Materials and methods

2.1. Preparation of human detrusor strips

Specimens from the anterior part of the urinary bladder dome were obtained from 38 male patients (72 ± 8 average age) undergoing total cystectomy due to a bladder base malignancy. Approvals for the use and shipment of human tissues were obtained from the Ethics Committee of the Hospital of Vercelli (Italy) in accordance with Italian legislation and patient informed consent.

The specimens were transported to the laboratory in oxygenated Krebs solution (composition in mM: NaCl 120, KCl 4.7, $MgSO_4$ 0.6, $NaHCO_3$ 25, KH_2PO_4 1.2, $CaCl_2$ 2.0, glucose 10) maintained at 5–6 °C. Muscle strips (20 mm long, 4 mm wide) that were epithelium-denuded to minimise the influence of non-neural sources of ACh and ATP were prepared and stored overnight at 4 °C. The strips were then isometrically mounted in 2 ml organ baths superfused with Krebs solution at 37 °C bubbled with 95% O_2 and 5% CO_2. The tissues were allowed to equilibrate for 45 min under a tension of 2 g. Electrical field stimulation (EFS) was applied *via* two platinum electrodes placed parallel to the preparation. Various preparations were obtained from a single surgical specimen. Each preparation was used for a single protocol.

2.2. Release experiments in human detrusor strips

The neuronal release of [^{3}H]-ACh was assessed according to the procedure previously described for the human bladder (D'Agostino et al., 2006). Briefly, the preparation was incubated for 45 min with [methyl-^{3}H] choline (92 kBq/ml) and stimulated with 8 s pulse trains at 20 Hz (0.2 ms duration, 60 V/cm, 60 s apart) to label ACh stores. At the end of the labelling period, the preparation was washed out for 120 min by superfusion at a constant rate of 2 ml/min (Minipulse 2HP8 flow inducer, Gilson Medical Electronics, Middleton, WI, USA).

Starting at the 121st min (zero time), the superfusion fluid was collected continuously in 3 min periods (6 ml samples). Hemicholinium-3 (10 μM) was present in the washout solution throughout the experiment to prevent choline uptake, and the α blocker phentolamine (1 μM) was administered 60 min prior to S_1. The strip was stimulated two times (S_1 and S_2) beginning 9 (S_1) and 54 (S_2) min after the zero time. [^{3}H]-outflow was evoked by 6 trains of 60 pulses delivered at 20 Hz (0.5 ms duration, 60 V/cm, 33 s apart). Aliquots (1 ml) of the superfusate were added to 3 ml of Ultima Gold (Packard BioScience, Groningen, The Netherlands), and the tritium content was measured using liquid scintillation spectrometry (Tri-Carb 2700TR, PerkinElmer, Shelton, CT, USA). Quench correction curves were established and external standardisation was used to determine the counting efficiency. Both the resting- and stimulation-evoked outflows of radioactivity were expressed in disintegrations per s (Becquerels) per gram of tissue (Bq/g). The increase in the release caused by stimulation was obtained from the difference between the total tritium outflow during 3 min of stimulation plus the following 12 min (stimulation outflow period) and that calculated in the spontaneous outflow. The decline in the spontaneous outflow was calculated by fitting a linear regression line to the values (expressed in Bq/g) of 3 min samples before and after the stimulation outflow period. The drugs were added 9 min (agonists) or 30 min (antagonists and toxins) before the onset of S_2. The pre-junctional effect of the drug was expressed as the S_2/S_1 ratio in comparison to the equivalent ratio obtained in the absence of the drug (control experiments).

2.3. Contractile assessment in human detrusor strips

The EFS effectiveness in producing neuronal cholinergic smooth muscle contractions was recorded with a force displacement transducer and displayed on a polygraph (Battaglia Rangoni, Bologna, Italy) that allowed in each experiment to simultaneously record both the pre- and postjunctional effects. The postjunctional effects evoked by EFS were assessed using the means of 6 peak contractions evoked by S_1 and S_2.

2.4. Data analysis

The values from individual experiments were averaged and the S.E.M. values were calculated. Concentration–response curves (CRCs) for the effects of agonists were constructed in the absence and presence of antagonists. Drug potency estimates were evaluated as $-\log EC_{50}$ values (negative log of the molar concentration producing a half-maximal effect) obtained *via* nonlinear curve fitting (GraphPad Prism, Version 3.02, GraphPad Software, San Diego, CA, USA).

The apparent affinity (pK_b) estimates were calculated according to the method of Kenakin (1982). Differences between the means were analysed with a one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. Values of $P < 0.01$ were considered statistically significant.

2.5. Chemical compounds

The following compounds were purchased: [methyl-^{3}H]-choline chloride (2.89 TBq/mmol) from PerkinElmer, Inc. (Boston, MA, USA); TTX, ω-conotoxin GVIA and N-[[3-[(2S)-2 hydroxy-3-[[2-[4[(phenylsulfonyl)amino]phenyl]ethyl]amino]propoxy] phenyl]methyl]-acetamide (L748,337) from Tocris Cookson Ltd. (Cabot Park Bristol, UK); Hexamethonium bromide, Hemicholinium-3, Phentolamine hydrochloride, (±)-Isoprenaline hydrochloride (INA), propranolol hydrochloride, [(±)-1-[2,3-(dihydro-7-methyl-1H-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol] hydrochloride (ICI118,55) and 1-(2-Ethylphenoxy)-3-[[(1S)-1,2,3,4-tetrahydro-1-naphthalenyl]amino]-(2S)-2-propanol hydrochloride (SR59230A), (±)-(R*,R*)-[4-[2-[[2-(3-Chlorophenyl)-2-hydroxyethyl]amino] propyl]phenoxy]acetic acid sodium hydrate (BRL 37344A); 5-[[[(3-phenoxyphenyl)methyl][(1S)-1,2,3,4-tetrahydro-1-naphthalenyl]-amino] carbonyl]-1,2,4-benzenetricarboxylic acid, sodium salt hydrate (A-317491) from Sigma-RBI (St. Louis, MO, USA); and 2-Amino-N-[4-[2-[[(2R)-2-hydroxy-2-phenylethyl]amino]ethyl] phenyl]-4-thiazoleacetamide (mirabegron) from Santa Cruz Biotechnoloy, Inc. (Santa Cruz, Ca, USA).