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RESEARCH PAPER

β_3 Adrenoceptor-induced cholinergic inhibition in human and rat urinary bladders involves the exchange protein directly activated by cyclic AMP 1 favoring adenosine release

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Background and Purpose: The mechanism by which β_3 receptor agonists (e.g. mirabegron) control bladder overactivity may involve adenosine release from human and rat detrusor smooth muscle. Retrograde activation of adenosine A_1 receptors reduces ACh release from cholinergic bladder nerves. β_3-Adrenoceptors usually couple to adenylyl cyclase. Here we investigated, which of the cAMP targets, protein kinase A or the exchange protein directly activated by cAMP (EPAC) could be involved in this cholinergic inhibition of the bladder.

Experimental Approach: [^{3}H]ACh and adenosine release from urothelium-denuded detrusor strips of cadaveric human organ donors and rats were measured by liquid scintillation spectrometry and HPLC, respectively. In vivo cystometry was also performed in urethane-anaesthetized rats.

Key Results: The exchange protein directly activated by cAMP (EPAC) inhibitor, ESI-09, prevented mirabegron- and isoprenaline-induced adenosine release from human and rat detrusor strips respectively. ESI-09, but not the PKA inhibitor, H-89, attenuated inhibition of [^{3}H]ACh release from stimulated (10 Hz) detrusor strips caused by activating β_3-adrenoceptors, AC (forskolin) and EPAC1 (8-CTP-2Me-cAMP). Isoprenaline-induced inhibition of [^{3}H]ACh release was also prevented by inhibitors of PKC (chelerythrine and Go6976) and of the equilibrative nucleoside transporter 1 (ENT1; dipyridamole and NBTI), but not by PLC inhibition with U73122. Pretreatment with ESI-09, but not with H-89, prevented the reduction of the voiding frequency caused by isoprenaline and forskolin in vivo.

Abbreviations: 1,9-ddFSK (1,9-dideoxyforskolin), 7β-acetoxy-6β-hydroxy-8,13-epoxy-labd-14-en-11-one; 8-CPT-2Me-cAMP, 8-(4-chlorophenylthio)-2′-*O*-methyladenosine-3′,5′-cyclic monophosphate sodium salt; 8-pCPT-2-O-Me-cAMP-AM (007-AM), 8-(4-chlorophenylthio)-2′-*O*-methyladenosine-3′,5′-cyclic monophosphate acetoxymethyl ester; ABT 702, 5-(3-bromophenyl)-7-[6-(4-morpholinyl)-3-pyrido[2,3-*d*]byrimidin-4-amine dihydrochloride; CHL, chelerythrine; CL316,243, 5-[(2*R*)-2-[[(2*R*)-2-(3-chlorophenyl)-2-hydroxyethyl]amino]propyl]-1,3-benzodioxole-2,2-dicarboxylic acid disodium salt; Dipy, dipyridamole; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; EFS, electrical field stimulation; ENT1, type 1 equilibrative nucleoside transporter; EPAC, exchange protein directly activated by cAMP; ESI-05, 1,3,5-trimethyl-2-[(4-methylphenyl)sulfonyl]benzene; ESI-09, α-[(2-(3-chlorophenyl)hydrazinylidene]-5-(1,1-dimethylethyl)-β-oxo-3-isoxazolepropanenitrile; Fenoterol, 2-(3,5-dihydroxyphenyl)-2-hydroxy-2′-(4-hydroxyphenyl)-1′-methyldiethylamine hydrobromide; FSK, forskolin; Go6976, 5,6,7,13-tetrahydro-13-methyl-5-oxo-12*H*-indolo[2,3-*a*]pyrrolo[3,4-*c*]carbazole-12-propanenitrile; H-89, *N*-[2-[[3-(4-bromophenyl)-2-propenyl]amino]ethyl]-5-isoquinolinesulfonamide dihydrochloride; L-748,337, *N*-[[3-[(2*S*)-2-hydroxy-3-[[2-[4-[(phenylsulfonyl)amino]phenyl]ethyl]amino] propoxy]phenyl]methyl]-acetamide; m-3M3FBS, 2,4,6-trimethyl-*N*-[3-(trifluoromethyl) phenyl]benzenesulfonamide; NBTI, *S*-(*p*-nitrobenzyl)-6-thioinosine; OAB, overactive bladder; PKA, protein kinase A; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; SR59230A, 1-(2-ethylphenoxy)-3-[[(1*S*)-1,2,3,4-tetrahydro-1-naphthalenyl]amino]-(2*S*)-2-propanol hydrochloride; U73122, 1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione.

Conclusion and Implications: Data suggest that β_3-adrenoceptor-induced inhibition of cholinergic neurotransmission in human and rat urinary bladders involves activation of an EPAC1/PKC pathway downstream cAMP production resulting in adenosine outflow via ENT1.

1 | INTRODUCTION

Selective **β_3-adrenoceptor** agonists, such as **mirabegron**, are increasingly prescribed as alternatives to anti-muscarinic drugs in the treatment of overactive bladder (OAB) syndrome (Angulo, Khullar, Nitti, & Siddiqui, 2013; Marcelissen, Rashid, Antunes-Lopes, Delongchamps, & Geavlete, 2019). Regardless of their clinical success and that they are associated with fewer side effects compared to anti-muscarinic drugs, controversy exists regarding the dominant localization of β_3-adrenoceptors on detrusor smooth muscle fibres and the molecular mechanism(s) underlying their overall mechanism (Andersson, 2017; Igawa & Aizawa, 2017; Igawa, Aizawa, & Michel, 2019; Okeke, Gravas, & Michel, 2017). While activation of the β_3-adrenoceptor was initially associated with direct detrusor relaxation (Igawa et al., 1999; Takasu et al., 2007; Takeda et al., 1999; Wuest et al., 2009), the peak plasma levels of mirabegron when administrated in therapeutic (50 mg daily) doses (83–167 nM) are below its effective concentration (EC_{50}~1–3 μM) to relax detrusor smooth muscle strips (Maki et al., 2019; Michel & Korstanje, 2016). This paradoxical finding has led to emergence of newer theories to explain the efficacy of β_3-adrenoceptor agonists at the low nanomolar range. This includes inhibition of bladder sensory input during the micturition cycle (Aizawa, Homma, & Igawa, 2015; Woods, Carson, Nortan, Sheldon, & Argentieri, 2001) and down-regulation of ACh release from bladder parasympathetic nerves (D'Agostino, Condino, & Calvi, 2015; Silva et al., 2017), thereby reducing the level of detrusor contraction.

In support of this mechanism, recent data from our group has demonstrated that activation of β_3-adrenoceptors causes the release of **adenosine** from urothelium-denuded human and rat detrusor strips via *S*-(*p*-nitrobenzyl)-6-thioinosine (**NBTI**)- and **dipyridamole** (Dipy)-sensitive equilibrative nucleoside transporter 1 (ENT1; Silva et al., 2017). Thus, cholinergic inhibition by β_3-adrenoceptor agonists may be indirectly mediated by adenosine released from detrusor smooth muscle fibres leading to retrograde activation of inhibitory **A_1 receptors** on cholinergic nerve terminals (Silva et al., 2017; Silva-Ramos et al., 2015). This new mechanism may be clinically relevant because, contrary to the initial view that normally there is no parasympathetic nerve drive during bladder storage, recent evidence demonstrates that there is the 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, Miyamae, Iwashita, Otani, & Inadome, 2004; reviewed in Winder, Tobin, Zupančič, & Romih, 2014). This release might be controlled by adenosine spillover from cells expressing β_3-adrenoceptors. However, it remains to be determined the exact mechanism causing the release of adenosine downstream β_3-adrenoceptors activation in the urinary bladder.

Mirabegron increases intracellular **cAMP** accumulation in CHO cells stably transfected with rat and human β_3-adrenoceptors (Hatanaka et al., 2013). Intracellular effects of cAMP are most often attributed to protein kinase A (PKA; Wang et al., 2017). However, detrusor relaxation owing to downstream activation of the AC/**PKA** pathway by β-adrenoceptors has been questioned (Frazier, Mathy, Peters, & Michel, 2005; Maki et al., 2019), raising the hypothesis that PKA-independent effects are also involved (de Rooij et al., 1998; Kawasaki et al., 1998; reviewed in Dekkers, Racké, & Schmidt, 2013). **The exchange protein directly activated by cAMP** (EPAC) is a cAMP-regulated guanine nucleotide exchange factor that favours GDP/GTP exchange and activation of small Ras-like GTPases (Li et al., 2007). This prompted us to investigate this cAMP-dependent pathway (e.g. PKA and/or EPAC), which would be the most likely mechanism involved in β_3-induced cholinergic inhibition in human and rat urinary bladders.

What is already known

- β_3 agonists may control detrusor overactivity indirectly by releasing adenosine via ENT1 membrane transporters.
- Retrograde activation of adenosine A1 receptors inhibits acetylcholine release in human and rat bladders;

What this study adds

- β_3-adrenoceptors co-localize with EPAC1 and ENT1on bladder smooth muscle fibres of human and rats;
- β_3-adrenoceptor-induced adenosine release involves activation of the EPAC1/PKC/ENT1 pathway downstream cyclic AMP accumulation;

Clinical significance

- β_3-adrenoceptor-induced rehabilitation of the adenosine A1 inhibitory tone may contribute to the therapeutic success;
- Manipulation of EPAC1/PKC/ENT1 activity may provide additional therapeutic targets to reduce cholinergic bladder overactivity.

2 | METHODS

2.1 | Animals

Animals care and experimental procedures were conducted in strict accordance with the recommendations of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS 123), Directive 2010/63/EU, and Portuguese rules (DL 113/2013). All experimental protocols involving animals were approved by the competent national authority Direção Geral de Alimentação e Veterinária and by the ICBAS Animal Ethical Committee (No. 224/2017); experimental design, data analysis, and reporting are in accordance to Curtis, Ashton, Moon, and Ahluwalia (2018). Animal studies are reported in compliance with the ARRIVE guidelines (Kilkenny, Browne, Cuthill, Emerson, & Altman, 2010) and with the recommendations made by the *British Journal of Pharmacology*. A total of 88 animals were used in the experiments described here, including both in vivo and in vitro. Male rats (Wistar, 200–300 g; Charles River, Barcelona, Spain; RGD Cat. No. 13508588, RRID:RGD_13508588) were kept at a constant temperature (21°C) and a regular light (06:30–19:30 hr)–dark (19:30–06:30 hr) cycle, with food and water provided ad libitum.

2.2 | Human bladder samples

Samples of the human detrusor were collected from the bladder dome of 18 male organ donors (38 ± 4 years of age) at the time of harvesting their organs for transplantation. Collected samples were immediately placed at 4–6°C in mannitol transplantation solution at 400 mOsm·kg^{-1} (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 hr 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 her or his life. The investigation conforms to the principles outline in *The Code of Ethics of the World Medical Association* (Declaration of Helsinki).

2.3 | Quantification of [^{3}H]ACh release

The experiments were performed on isolated detrusor muscle strips without the mucosa for 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 for human and rat bladder samples respectively (Carneiro et al., 2014; Silva et al., 2017; Silva-Ramos et al., 2015). Full thickness isolated detrusor muscle strips (3 mm width, 5 mm length; weighting 9.2 ± 0.5 mg [human] and 5.9 ± 0.2 [rat]) were mounted in 365-μl capacity chambers of a Brandel SF-12 automated superfusion system (Valley International Corp., Austin, TX, USA) heated at 37°C. 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 over 40 min with 1-μM [^{3}H]choline (specific activity 5 μCi·nmol^{-1}) under electrical field stimulation (EFS, 1-Hz frequency, 0.5-ms pulse width, 75 mA) using two platinum-made grid electrodes placed above and below the muscle strip (transmural EFS). Following loading, the washout superfusion (1 ml·min^{-1}) 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 square wave pulses of 0.5-ms duration delivered at 10-Hz frequency. Test drugs were added 8 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 each stimulation period (S_1 and S_2); the values obtained are used to calculate S_2/S_1 ratios (see, e.g., Carneiro et al., 2014; Silva et al., 2017). In the absence of test drugs, S_2/S_1 ratios were 0.99 ± 0.04 (n = 6) and 0.96 ± 0.02 (n = 5) in human and rat detrusor samples, respectively. None of the test drugs and their modifiers changed significantly (P > .05) the basal [^{3}H]ACh release.

2.4 | Measurement of adenosine release

The procedures used to measure the release of adenine nucleosides (adenosine and inosine) were previously described (see 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 and 30 min after starting the experimental protocol (zero time). The β_3-adrenoceptor agonists (**isoprenaline**, 1 μM; mirabegron, 0.1 μM; and 5-[(2*R*)-2-[[(2*R*)-2-(3-chlorophenyl)-2-hydroxyethyl]amino]propyl]-1,3-benzodioxole-2,2-dicarboxylic acid disodium salt [**CL316,243**], 1 μM), as well as the EPAC1 activators (8-(4-chlorophenylthio)-2′-*O*-methyladenosine-3′,5′-cyclic monophosphate sodium salt [**8-CPT-2Me-cAMP**], 20 μM and 8-(4-chlorophenylthio)-2′-*O*-methyladenosine-3′,5′-cyclic

monophosphate acetoxymethyl ester [8-pCPT-2-*O*-Me-cAMP-AM], 20 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); EFS was not used in these experiments. Bath aliquots (50–250 μl) were frozen in liquid nitrogen immediately after collection, stored at −20°C (the enzymes are stable for at least 4 weeks), and at least two replicates analysed 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; RRID:SCR_014593). Chromatographic separation was carried out through a Hypersil GOLD C15 column (5 μM, 2.1 mm × 150 mm) equipped with a guard column (5 μm, 2.1 mm × 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 $\mu l \cdot min^{-1}$ and the column temperature was maintained at 20°C. The autosampler was set at 4°C 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.

2.5 | Immunofluorescence staining and confocal microscopy observation

Human and rat detrusor strips without the mucosa were stretched to all directions and pinned onto a Petri dish coated with Sylgard®. The strips were then fixed in PLP solution (paraformaldehyde 2%, lysine 0.075 M, sodium phosphate 0.037 M, and sodium periodate 0.01 M) for 16 hr at 4°C. Sixteen-micron sections were incubated with selected primary antibodies (Table 1) diluted in an incubation buffer (fetal bovine serum 5%, serum albumin 1% and Triton X-100 0.3% in PBS), at 4°C, for 16 hr. 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 1) in the dark for 2 hr at room temperature. The human hippocampus (DG, dentate gyrus) and the rat aorta were used as positive controls for the **β_2-adrenoceptor** immunoreactivity; the same antibody dilution (1:50, AAR-016) and immunostaining procedure was as mentioned above. Cadaveric human hippocampal samples were previously collected and used as described by our group (Barros-Barbosa et al., 2016). 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 (RRID:AB_2336789) as antifade mounting media (VectorLabs; RRID:SCR_000821) and stored in the dark at 4°C. Observations were performed and analysed with a laser-scanning confocal microscope (Olympus FluoView, FV1000, Tokyo, Japan; RRID:SCR_016840) and analysed with the Fluoview FV10-ASW software, RRID:SCR_014215.

2.6 | 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%, SDS 0.1%, and 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 70°C for 10 min, subjected to electrophoresis in 10% SDS-PAGE and electrotransferred onto PVDF membranes (MilliPore, MA, USA). Protein load was 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 BSA 5% and, subsequently, incubated either with the following primary antibodies: rabbit anti-β_2 (AAR-016; 1:200) and anti-β_3 (AAR-017; 1:200) adrenoceptor primary antibodies from Alomone Labs (Jerusalem, Israel) or with rabbit anti-EPAC1 (ab21236; 1:100; Abcam, Cambridge, UK) and mouse anti-EPAC2 (5B1, mAb #4156; 1:500; Cell Signaling Technology, Danvers, MA, USA), in the above blocking buffer overnight at 4°C. Membranes were washed three times for 10 min in TBS/Tween-20 and incubated with HRP-conjugated secondary antibody (1:25,000; Abcam) for 1 hr at room temperature. For normalization purpose, membranes were incubated either with mouse anti-GAPDH (EC 1.2.1.12) primary antibody from Santa Cruz (1:200, SC-32233, Dallas, TX, USA) or with rabbit anti-β-actin from Abcam (1:1,000, ab8227) 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). The immuno-related procedures used comply with the recommendations made by the *British Journal of Pharmacology* (Alexander et al., 2018).

2.7 | In vivo cystometry

The experiments were carried out in urethane anaesthetized rats (1.0–1.2 $g \cdot kg^{-1}$), spontaneously breathing. Core body temperature was kept between 36°C and 38°C 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 $ml \cdot hr^{-1} \cdot kg^{-1}$) and i.v. 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 and the

TABLE 1 Primary and secondary antibodies used to stain human and rat detrusor strips

Antigen	Code/identifier	Host	Dilution	Supplier
Primary antibodies				
Anti-β_2 receptor	AAR-016/RRID:AB_2039718	Rabbit	1:50	Alomone
Anti-β_3 receptor	AAR-017/RRID:AB_2039720	Rabbit	1:50	Alomone
Anti-β_3 receptor	MC-4198/RRID:AB_590525	Rabbit	1:50	MBL International
Anti-EPAC1	ab21236/RRID:AB_2177464	Rabbit	1:75	Abcam
Anti-EPAC1	SC-28366/RRID:AB_627521	Mouse	1:100	Santa Cruz
Anti-EPAC2	#4156/RRID:AB_1904112	Mouse	1:50	Cell Signaling Tech
Anti-ENT1	ANT-051/RRID:AB_2341015	Rabbit	1:50	Alomone
Secondary antibodies				
Alexa Fluor 488 anti-rabbit	A-21206/RRID:AB_2535792	Donkey	1:1,000	Molecular Probes
Alexa Fluor 633 anti-mouse	A-21050/RRID:AB_2535718	Goat	1:1,000	Molecular Probes

TABLE 2 Mechanism of action of drugs used in this study

Drug	Mechanism of action
Isoprenaline	β-adrenoceptor agonist
Fenoterol	Selective β_2-adrenoceptor agonist
Mirabegron	Selective β_3-adrenoceptor agonist (human)
CL316,243	Selective β_3-adrenoceptor agonist (rodent)
L-748,337	Selective β_3-adrenoceptor antagonist (human)
SR59230A	β_3-adrenoceptor antagonist (rodent)
DPCPX	Selective adenosine A_1 receptor antagonist
Dipyridamole (Dipy)	ENT transport inhibitor
S-(*p*-nitrobenzyl)-6-thioinosine (NBTI)	ENT1 transport inhibitor
ABT 702	Adenosine kinase inhibitor
Forskolin (FSK)	AC activator
1,9-dideoxyforskolin (1,9-ddFSK)	AC inactive analogue of FSK
H-89	PKA inhibitor
Rp-cAMPS	Selective PKA inhibitor (cell permeable)
ESI-09	EPAC inhibitor
ESI-05	Selective EPAC2 inhibitor
8-CPT-2Me-cAMP	EPAC1 activator
8-pCPT-2-O-Me-cAMP-AM	EPAC1 activator (cell permeable)
m-3M3FBS	PLC activator
U73122	PLC inhibitor
Phorbol 12-myristate 13-acetate (PMA)	PKC activator
Chelerythrine (CHL)	PKC inhibitor
Go6976	PKC inhibitor (Ca^{2+}-dependent PKCα and PKCβ1 isoforms)

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; RRID:SCR_001620), which was also used to record haemodynamic 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^{-1} 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^{-1}), which is within the range used in previous studies to obtained stable micturition cycles during continuous cystometrograms in anaesthetized rats (e.g. Carneiro et al., 2014). The

flow rate was twofold to fourfold higher than the normal urinary debit in experimental rats (15–30 ml·day^{-1}) and are similar to that 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. For the sake of clarity, the results presented in this study will consider the per cent variation of intercontraction interval (ICI, min) values as compared to the control situation achieved after six consecutive voiding contractions of similar amplitude. Test drugs were applied into the bladder lumen by changing the syringe connected to the automate perfusion pump (0.04 ml·min^{-1}).

2.8 | Drugs and solutions

Choline chloride; 1,3-dipropyl-8-cyclopentylxanthine (**DPCPX**); α-[2-(3-chlorophenyl)hydrazinylidene]-5-(1,1-dimethylethyl)-*b*-oxo-3-isoxazolepropanenitrile (**ESI-09**); **fenoterol** hydrobromide; 7β-acetoxy-8,13,epoxy-1α,6β,9α-trihydroxylabd-14-en-11-one (**Forskolin**, FSK); 1,9-dideoxyforskolin (1,9-ddFSK); hemicholinium-3 (HC-3); **Rp-adenosine 3′,5′-cyclic monophosphorothioate triethylammonium salt**; and NBTI were obtained from Sigma (St Louis, MO, USA; RRID:SCR_008988). 5-(3-Bromophenyl)-7-[6-(4-morpholinyl)-3-pyrido[2,3-*d*]byrimidin-4-amine dihydrochloride (**ABT 702**); CL316,243; *N*-[2-[[3-(4-bromophenyl)-2-propenyl]amino]ethyl]-5-isoquinoline sulfonamide dihydrochloride (**H-89**); chelerythrine (**CHL**); 8-CPT-2Me cAMP; 8-pCPT-2-*O*-Me-cAMP-AM (007-AM); 1,3,5-trimethyl-2-[(4-methylphenyl) sulfonyl] benzene (ESI-05); 1-(2-ethylphenoxy)-3-[[(1*S*)-1,2,3,4-tetrahydro-1-naphthalenyl]amino]-(2*S*)-2-propanol hydrochloride (**SR59230A**); isoproterenol hydrochloride (isoprenaline); 2,4,6-trimethyl-*N*-[3-(trifluoromethyl)phenyl] benzenesulfonamide (**m-3M3FBS**); 1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione (**U73122**); phorbol 12-myristate 13-acetate (**PMA**); and 5,6,7,13-tetrahydro-13-methyl-5-oxo-12*H*-indolo[2,3-*a*] pyrrolo[3,4-*c*]carbazole-12-propanenitrile (**Go6976**) were obtained from Tocris Bioscience (Bristol, UK; RRID:SCR_003689). *N*-[[3-[(2*S*)-2-hydroxy-3-[[2-[4-[(phenylsulfonyl)amino]phenyl]ethyl]amino] propoxy]phenyl]methyl]-acetamide (**L-748,337**) was obtained from Santa Cruz (RRID:AB_2629503). Mirabegron was obtained from Selleckchem.com (Houston, USA). Dipy 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% DMSO + 1% NaOH 1 M (v/v). ABT 702, Dipy, 8-pCPT-2-O-Me-cAMP-AM, ESI-05, ESI-09, FSK, 1,9-ddFSK, Go6976, L-748,337, m-3M3FBS, mirabegron, NBTI, PMA, and U73122 were dissolved in DMSO. Other drugs were prepared in Tyrode's solution. All stock solutions were stored as frozen aliquots at −20°C. Dilutions of these stock solutions were made daily and appropriate solvent controls were done. No 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. The mechanism of action of drugs used in this study is shown in Table 2.

2.9 | 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; no outliers were excluded from data analysis and presentation; and the *n* number was always equal or higher than 5. Only one experimental procedure (e.g. agonist in the absence and in the presence of the antagonist/enzymatic inhibitor) was performed in vivo per individual; different in vitro experimental protocols were performed in parallel from separated samples of the same bladder to reduce the number of individuals. No experimental replicates (identical protocols) were made using the same biological specimen. Accounting for experimental failures each protocol was repeated at least in seven tissue samples/individuals. Statistical analysis of data was carried out using GraphPad Prism 7.04 for Windows software (La Jolla, USA; RRID: SCR_002798). One- or two-way ANOVA followed by the Dunnett's multicomparison test was used only if F was significant and there was no variance inhomogeneity. $P < .05$ (two-tailed) values were considered statistically significant. The data and statistical analysis comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology (Curtis et al., 2018).

2.10 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander, Christopoulos, et al., 2019; Alexander, Fabbro, et al., 2019; Alexander, Kelly, et al., 2019).

3 | RESULTS

3.1 | β_3-Adrenoceptors activation triggers adenosine outflow via ENT1 through downstream intracellular activation of EPAC1

Mirabegron (0.1 μM) and isoprenaline (1 μM) promote adenosine outflow from non-stimulated urothelium-denuded human and rat detrusor strips, without significantly affecting the release of inosine (Figure 1a$_i$,b$_i$). The involvement of β_3-adrenoceptors was confirmed in the human bladder as mirabegron (0.1 μM)-induced adenosine release was attenuated by the selective β_3-adrenoceptor antagonist L-748,337 (30 nM, Figure 1a$_{ii}$), a compound that has a 100-fold higher affinity (K_i~4 nM) for the β_3 over β_1 and β_2 adrenoceptors

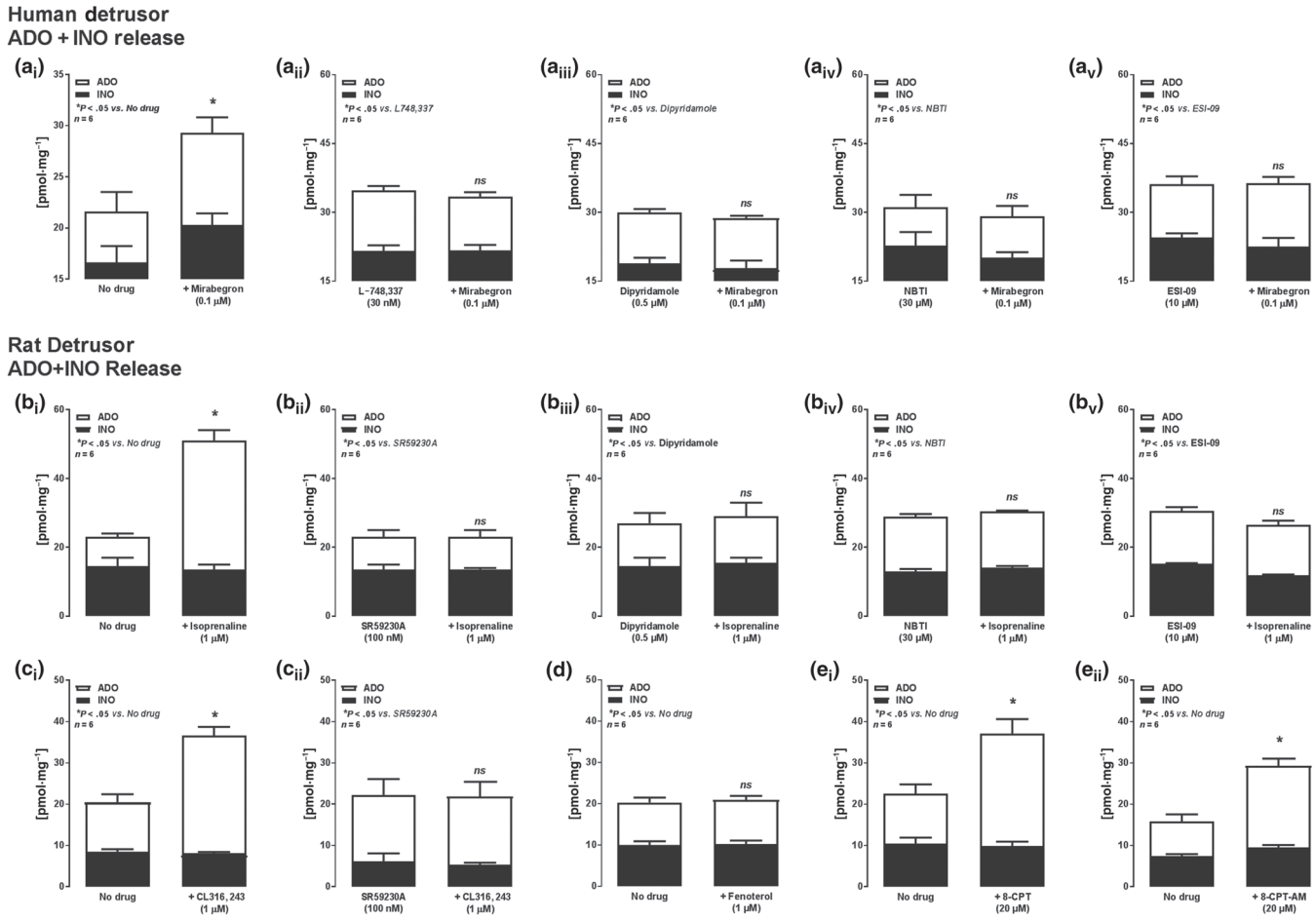

FIGURE 1 Effects of mirabegron (0.1 μM, a_{i-iv}), isoprenaline (Isop, 1 μM, b_{i-iv}), CL316,243 (1 μM, c_{i-ii}), fenoterol (1 μM, d), 8-CPT-2Me cAMP (20 μM, e_i), and 8-pCPT-2-O-Me-cAMP-AM (20 μM, e_{ii}) on adenosine outflow from urothelium-denuned human (a) and rat (b–e) detrusor strips, respectively, in the absence and in the presence of β_3-adrenoceptors antagonists (L-748,337, 30 nM; SR592230A, 100 nM) and of inhibitors of ENT1 (dipyridamole, 0.5 μM; NBTI, 30 μM) and EPAC (ESI-09, 10 μM). Mirabegron, isoprenaline, CL316,243, fenoterol, 8-CPT-2Me cAMP, and 8-pCPT-2-O-Me-cAMP-AM contacted with the preparations for 15 min before sample collection. Antagonists/inhibitors were applied 15 min before mirabegron, isoprenaline or CL316,243 and were maintained throughout the assay. The ordinates represent the amount of adenosine (ADO, white bars) and inosine (INO, black bars) in pmol·mg^{-1} of wet weight of the preparations detected by HPLC with diode array in samples collected from the incubation media at 15 min intervals (for details, see Section 2). Data are means ± SD of five to eight individuals; duplicates were performed for each individual experiment. $^*P < .05$ (two-way ANOVA followed by the Sidak's multiple comparison test) represent significant differences when compared to the control situation (basal/inhibitor alone)

and shoes preference for the human over the rat β_3 adrenoceptor (Candelore et al., 1999; Cernecka, Sand, & Michel, 2014). A similar blocking effect of isoprenaline (1 μM)-induced adenosine release was observed in rat detrusor strips with SR59230A (100 nM, Figure 1b_{ii}). This compound exhibits reasonably high affinity for the rat β_3-adrenoceptor (K_i values of 40, 408 and 648 nM for β_3-, β_1- and β_2-adrenoceptors, respectively; Kanzler, Januario, & Paschoalini, 2011) but does not discriminate well between stably transfected human β-adrenoceptor subtypes compared to L-748,337 (Baker, 2010; Candelore et al., 1999; Hoffmann, Leitz, Oberdorf-Maass, Lohse, & Klotz, 2004; Niclauss, Michel-Reher, Alewijnse, & Michel, 2006; reviewed in Schena & Caplan, 2019). Notwithstanding this, involvement of β_3-adrenoceptors is endorsed by the fact that isoprenaline (1 μM)-induced adenosine release was reproduced by the rodent selective β_3-adrenoceptor agonist, CL316,243 (1 μM; Figure 1c_i; Evans, Papaioannou, Hamilton, & Summers, 1999), but not by the β_2-adrenoceptor agonist, fenoterol (1 μM; Figure 1d). Facilitation of adenosine release by CL316,243 (1 μM) was also attenuated after pretreatment with SR59230A (100 nM, Figure 1c_{ii}).

Pretreatment with ENT inhbitors, either Dipy (0.5 μM) or NBTI (30 μM), also prevented the β_3-adrenoceptor-induced adenosine outflow in both species (Figure 1a_{iii-iv},b_{iii-iv}), while NBTI is a potent and selective ENT1 inhibitor, Dipy non-selectively inhibits ENT transporters and also has affinity for PDE enzymes (Parkinson, Xiong, & Zamzow, 2005).

Pre-incubation of urothelium-denuded human (Figure 1a_v) and rat (Figure 1b_v) detrusor strips with the EPAC inhibitor, ESI-09 (10 μM), also abolished mirabegron (0.1 μM)- and isoprenaline (1 μM)-induced adenosine release. The ability of ESI-09 to selectively and

competitively inhibit EPAC activity in concentrations below 20 μM has been demonstrated (Zhu et al., 2015) both in vitro (Almahariq et al., 2013) and in vivo (Almahariq et al., 2014; Gong et al., 2013). Selective activation of EPAC1 with 8-CPT-2Me-cAMP (20 μM, Figure 1e$_i$) and its cell permeable acetoxymethyl ester analogue (8-pCPT-2-O-Me-cAMP-AM, 20 μM, Figure 1e$_{ii}$; Vliem et al., 2008) also stimulated the release of adenosine mimicking the effects of isoprenaline (1 μM; Figure 1b$_i$) and CL316,243 (1 μM; Figure 1c$_i$) in rat detrusor strips.

3.2 | Human and rat detrusor samples are immunoreactive for the β_3-adrenoceptor, EPAC1 and ENT1 but express very small amounts of the β_2-adrenoceptor and EPAC2

Using immunofluorescence confocal microscopy, we show that human and rat detrusor samples exhibit significant immunoreactivity against β_3-adrenoceptor (Figure 2a), EPAC1 (Figure 2c) and ENT1 (Figure 2e) proteins. β_3-Adrenoceptor (Figure 2a$_{iv}$) and ENT1 (Figure 2e$_{iv}$) immunoreactivities follow a plasma membrane staining pattern, whereas EPAC1 is compatible with its diffuse cytoplasmic distribution. These findings contrast with faint immunoreactivity signals obtained for β_2-adrenoceptor (Figure 2b) and EPAC2 (Figure 2d) proteins in urothelium-denuded human and rat detrusor samples. The lack of β_2-adrenoceptor (Figure 2g) and EPAC2 (Figure 2h) immunofluorescence staining in the detrusor was confirmed by Western blot analysis and cannot be attributed to the quality of the antibodies. Positive identification of the β_2-adrenoceptor was previously demonstrated using the same antibody in the mouse aorta (Moreira-Rodrigues et al., 2014) and was further confirmed here using the rat aorta and the dentate gyrus of the human hippocampus (Figure S1). Regarding the EPAC2 antibody from Cell Signaling Tech, it was recently validated in the knockout mouse (Hwang et al., 2017). Moreover, both AAR-016 and AAR-017 antibodies detected major bands with apparent sizes around 44 kDa, which are compatible with the theoretical molecular masses of human and rat β_2 (~46 kDa) and β_3 (~43 kDa) adrenoceptors (Figure 2g). Although the SC-28366 detected a stronger band in the rat than in the human detrusor, migration of these bands stopped in the proximity of the 100-kDa ladder marker, which is compatible with the theoretical molecular mass (~104 kDa) of the EPAC1 protein (Figure 2h). A very faint band was detected with the CellS4156 antibody in the rat, but not in the human, at the predicted molecular mass of EPAC2 (116 kDa).

To ascertain that EPAC and β_3-adrenoceptors co-localize in detrusor smooth muscle fibres, we used distinct commercially available antibodies raised in mice and rabbits respectively (Figure 2f). The antibody from MBL International Corporation (MC4198, also known as LSA4198) was raised against a synthetic 20 amino acid peptide from the N-terminal domain of the human β_3-adrenoceptor and shows no homology with any other human protein as determined by BLAST (Basic Local Alignment Search Tool) analysis. The MC4198 antibody has been used successfully to detect this receptor in human (Limberg et al., 2010; Silva et al., 2017) and rat (Kullmann et al., 2009) bladders. This antibody differs from the one from Alomone Labs (AAR-007) used in Figure 2a, which is directed against a 13 amino acid peptide of the second extracellular loop of the mouse β_3-adrenoceptor showing 12/13 and 11/13 homology with the rat and human receptors, respectively, favouring cross-reactivity of this antibody among species. Concordant staining of human tissues with two or more antibodies (e.g. MC4198 and AAR-007) provides the most compelling evidence for β_3-adrenoceptor labelling in immunohistochemistry studies (Cernecka, Ochodnicky, Lamers, & Michel, 2012; Silva et al., 2017; reviewed in Igawa et al., 2019). As for the EPAC immunostaining, we used a rabbit polyclonal antibody directed against amino acids 526-541 of EPAC1 (Abcam, Ab21236; Figure 2c) and a mouse monoclonal antibody raised against amino acids 1-70 of the human EPAC1 (Santa Cruz, SC-28366; Figure 2f). The immunostaining pattern was concordant for both EPAC1 antibodies in the human detrusor. Figure 2f shows that the β_3-adrenoceptor co-localizes with EPAC1 on detrusor smooth muscle fibres, thus supporting the involvement of EPAC1 on adenosine outflow (via ENT1 transporters) triggered by β_3-adrenoceptors activation in the human urinary bladder.

3.3 | β_3-Adrenoceptors decrease nerve-evoked ACh release through a mechanism depending on EPAC1 activation and adenosine outflow via ENT1

Figure 3a shows that mirabegron, used in a concentration (0.1 μM) that selectively activates β_3-adrenoceptors (Takasu et al., 2007) and promotes adenosine outflow (Figure 1a) from the non-stimulated human detrusor, subsequently decreased by 51 ± 6% (n = 7) [^{3}H]ACh release from urothelium-denuded detrusor strips stimulated electrically (see also Figure 3b). The inhibitory effect of mirabegron was concentration dependent; it decreased nerve-evoked [^{3}H]ACh release by 30 ± 10% (n = 5) and 55 ± 10% (n = 5) when applied in 10 nM and 1 μM concentrations respectively. Likewise, isoprenaline (1 μM) decreased by 39 ± 1% (n = 6) the evoked [^{3}H]ACh release from rat detrusor strips without the mucosa (Figure 3c). Pharmacological evidence for the involvement of the β_3-adrenoceptor subtype in the inhibitory actions of mirabegron and isoprenaline on evoked [^{3}H]ACh release from human and rat detrusor strips, respectively, was recently provided by our group using selective β_2- and β_3-adrenoceptor agonists (fenoterol and CL316,243, respectively) and antagonists (SR59230A and ICI118,551, respectively; Silva et al., 2017). Increasing the concentration of fenoterol from 50 nM (Silva et al., 2017) to 1 μM (this study) did not significantly (P > .05) decreased nerve-evoked [^{3}H]ACh release from rat detrusor strips evaluated by the S_2/S_1 ratio (0.93 ± 0.05, n = 7 with fenoterol vs. 0.96 ± 0.02, n = 5 in control conditions).

Inhibition of the cAMP/EPAC pathway with ESI-09 (10 μM) attenuated mirabegron (0.1 μM)- and isoprenaline (1 μM)-induced

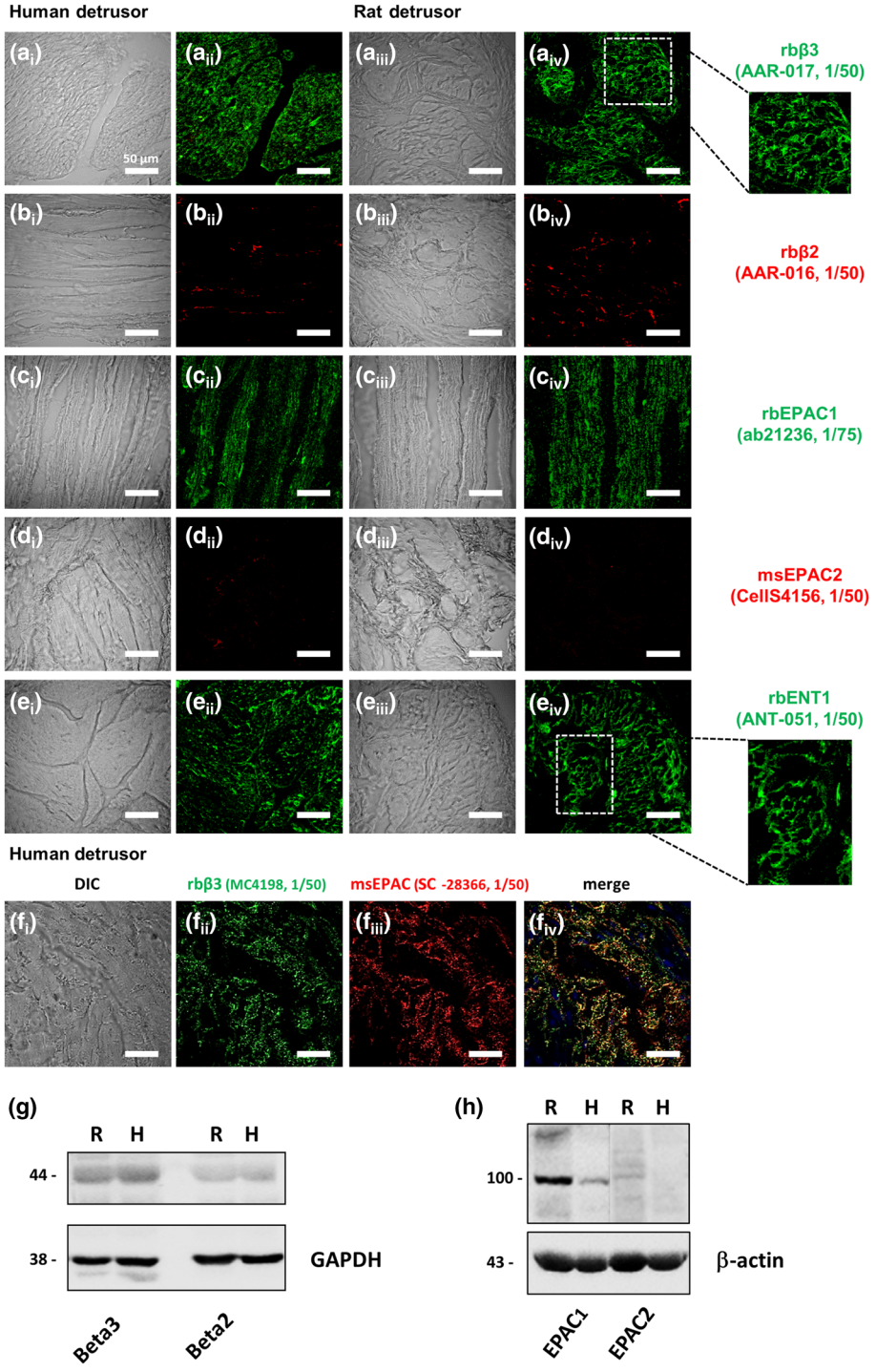

FIGURE 2 Immunolocalization of (a) β_3- and (b) β_2-adrenoceptors, (c) EPAC1 and (d) EPAC2 isoforms, and (e) ENT1 in transverse sections of the human (left hand-side panels) and rat (right hand-side panels) detrusor by confocal microscopy. β_3-Adrenoceptor and ENT1 immunoreactivities follow a plasma membrane staining pattern, whereas EPAC1 is compatible with a diffuse cytoplasmic distribution. Two distinct β_3 (AAR-017 and MC-4198 raised in rabbits) and EPAC (ab21236 and SC-28366 raised in rabbits and mice, respectively) antibodies were used as indicated. (f) Micrographs show that β_3-adrenoceptors (green) and EPAC (red) co-localize in human detrusor, as identified by the yellow staining in the merge image shown in the right hand-side image. Images are representative of four different individuals. Differential interference contrast (DIC) images are shown for comparison. Scale bar = 50 µm. Bottom panels are representative immunoblots of β_2- and β_3-adrenoceptors (g) and EPAC1 and EPAC2 isoforms (h) in urothelium-denuded detrusor homogenates of one male human subject (H) and one rat (R) run in parallel using the same antibodies as for the immunohistochemical staining. The highly conserved GAPDH enzyme (EC 1.2.1.12, MW~37 kDa) and β-actin (MW~43 kDa) were used as reference proteins for β_adrenoceptors and EPAC respectively. Images are representative of three different individuals. Gels were loaded with 150 µg of protein. Host species for antibody production were rabbit (rb) and mouse (ms)

decreases of evoked [^{3}H]ACh release from isolated human (Figure 3b) and rat (Figure 3c) detrusor strips respectively. There are two isoforms of EPAC, EPAC1 and EPAC2, produced by independent genes in mammals. While EPAC1 is ubiquitously expressed in all tissues (reviewed in Cheng, Ji, Tsalkova, & Mei, 2008), only minute amounts of EPAC2 were found in human and rat detrusor samples (see Figure 2d,h). This may explain why isoprenaline (1 µM)-induced inhibition of evoked [^{3}H]ACh release from the rat detrusor (S_2/S_1 ratio 0.57 ± 0.07, n = 6) was still observed in the presence of the selective EPAC2 inhibitor, ESI-05 (10 µM; S_2/S_1 ratio 0.54 ± 0.04, n = 5; P > .05), thus indicating that EPAC1 is the main isoform involved in β_3-adrenoceptor-induced cholinergic inhibition in the detrusor.

Figure 3b,c also shows that PKA inhibition with H-89 (10 µM) had no significant effect on mirabegron (0.1 µM)- and isoprenaline (1 µM)-induced inhibition of [^{3}H]ACh release from stimulated human and rat detrusor preparations respectively. Likewise, Rp-adenosine 3′,5′-cyclic monophosphorothioate triethylammonium salt (10 µM), which is a

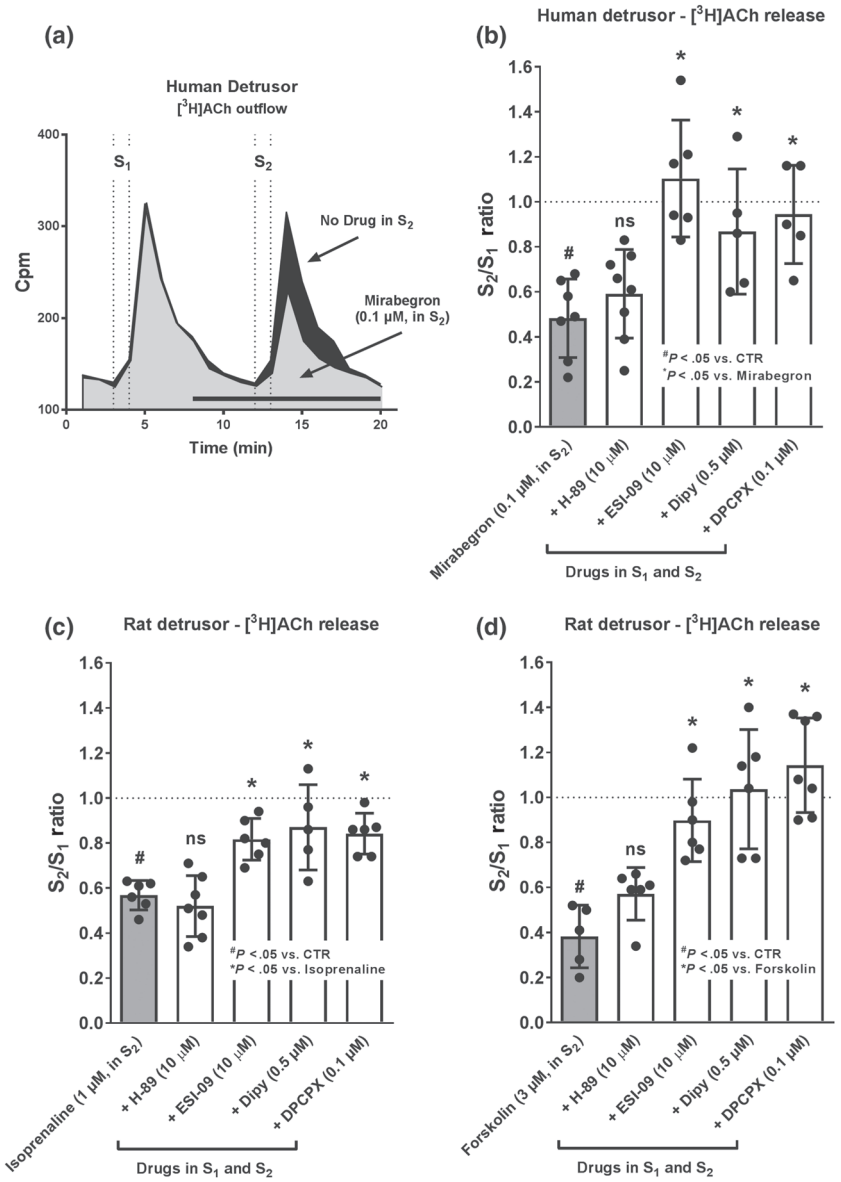

FIGURE 3 (a) Inhibitory effect of mirabegron on electrically evoked [^{3}H]ACh release from urothelium-denuded human detrusor strips. Ordinates represent tritium outflow expressed in scintillations per min (cpm). Abscissa indicates the times at which samples were collected. [^{3}H]ACh release was elicited 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). Mirabegron (0.1 μM) was added to the incubation media 6 min before S_2 (black horizontal bar). Panels b, c and d show the inhibitory effects of mirabegron, isoprenaline and forskolin on evoked [^{3}H]ACh release human (b) and rat (c and d) detrusor strips respectively. 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 of selective inhibitors of PKA (H-89, 10 μM), EPAC (ESI-09, 10 μM), and ENT1 (Dipy, 0.5 μM), as well as of the adenosine A_1 receptor antagonist, DPCPX (0.1 μM). All inhibitors and A_1 receptor antagonist 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 an *n* number of individuals (black dots). $^{\#,*}P < .001$ (one-way ANOVA followed by the Dunnett's multicomparison test with a single pooled variance) represent significant differences when compared to the control situation and to the inhibitory effects of mirabegron (b), isoprenaline (c) and forskolin (d) applied alone respectively

specific competitive inhibitor of PKA (IC_{50} = 4.9 μM) exhibiting high cell permeability and full resistance to cyclic nucleotide PDEs (Bell & McDermott, 1994), also failed (S_2/S_1 ratio 0.58 ± 0.02, *n* = 6; *P* > .05) to modify the inhibitory effect of isoprenaline (1 μM, S_2/S_1 ratio 0.57 ± 0.07, *n* = 6) on evoked [^{3}H]ACh release from rat detrusor strips. These negative results contrast with the actions of both PKA inhibitors on evoked [^{3}H]ACh release from rat myenteric neurons (Vieira, Duarte-Araújo, Adães, Magalhães-Cardoso, & Correia-de-Sá, 2009) and motor nerve terminals (Oliveira & Correia-de-Sá, 2005).

Figure 3d shows that ESI-09 (10 μM), but not H-89 (10 μM), significantly decreased the inhibitory effect of FSK (3 μM), a direct activator of the catalytic subunit of AC that on its own decreased evoked [^{3}H]ACh release by 61 ± 7% (*n* = 5) mimicking the inhibitory effect of β_3-adrenoceptors activation with isoprenaline (1 μM) in rat detrusor strips (Figure 3c). The involvement of cAMP on transmitter release inhibition by FSK (3 μM) is suggested because the inhibitory effect of its inactive analogue on AC, 1,9-ddFSK (3 μM), on [^{3}H]ACh release from stimulated rat detrusor strips did not exceed 10%, that is, the S_2/S_1 ratio changed from 0.96 ± 0.02 (*n* = 5) in control conditions to 0.86 ± 0.03 (*n* = 9) in the presence of 1,9-ddFSK (3 μM).

The involvement of adenosine release via ENT1 transporters indirectly mediating β_3-adrenoceptor inhibition of evoked [^{3}H]ACh release through the activation of prejunctional A_1 receptors on bladder cholinergic nerves (Silva et al., 2017; Silva-Ramos et al., 2015) was confirmed further in the present study. Figure 3 shows that transmitter release inhibition caused by mirabegron (0.1 μM, Figure 3b), isoprenaline (1 μM, Figure 3c), and FSK (3 μM, Figure 3d) was prevented both by the ENT transport inhibitor, Dipy (0.5 μM), and by the selective adenosine A_1 receptor antagonist, 1,3-dipropyl-8-cyclopentylxantine (DPCPX;100 nM).

The preventive effect of Dipy (0.5 μM, S_2/S_1 ratio 1.04 ± 0.09, n = 6) on FSK (3 μM)-induced inhibition of [^{3}H]ACh release from stimulated rat detrusor strips was mimicked by NBTI (30 μM, S_2/S_1 ratio 0.95 ± 0.11, n = 7), which unlike Dipy is a more selective ENT1 inhibitor with no affinity for PDE enzymes (Parkinson et al., 2005). This trend mimicked that obtained when isoprenaline (1 μM) was used instead for FSK (3 μM) in rat detrusor strips (Silva et al., 2017).

3.4 | β_3-Adrenoceptor-induced AC activation decreases the micturition frequency in anaesthetized rats through an EPAC1-dependent PKA-independent mechanism

Instillation of FSK (0.01–10 μM) into the bladder lumen of anaesthetized rats concentration-dependently decreased the micturition frequency (Figure 4aA); the latter was evidence by prolongation of the intercontraction interval (ICI, pEC_{50}–6.75 ± 0.30; E_{max} 39.30 ± 4.38%; n = 5) without modifying the amplitude (A) and the duration (ΔT) of voiding bladder contractions (Figure 4b$_i$). The AC activator mimicked the inhibitory effect of the β_3-adrenoceptor agonist, isoprenaline (0.1–1,000 nM), in the bladder of anaesthetized rats (Figure 4b$_{ii}$; cf. Silva et al., 2017). 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) found by others in the rat isolated detrusor (Yamazaki et al., 1998). Involvement of β_3-adrenoceptors in the inhibitory effect of isoprenaline on distension-induced micturition reflex in the anaesthetized rat was previously confirmed by our group using selective β_2- and β_3-adrenoceptor agonists (fenoterol and CL316,243, respectively) and antagonists (SR59230A and ICI118,551, respectively; Silva et al., 2017).

The inhibitory effects of FSK (0.01–10 μM) and isoprenaline (0.1–1,000 nM) on the micturition reflex seem to be mediated by targets located in the urinary bladder, since instillation of these drugs into the bladder lumen did not affect heart rate in the anaesthetized rat, which could have happened if drugs entered the systemic circulation or were applied via i.v. or i.p., that is, the variations of heart rate produced by FSK (445 ± 93 bpm vs. 464 ± 70 bpm with saline, n = 5) and isoprenaline (405 ± 52 bpm vs. 432 ± 36 bpm with saline, n = 5) at maximal concentrations were not significantly different (P > .05) from the control (saline) situation.

The participation of adenosine outflow via ENT1 transporters and subsequent activation of inhibitory A_1 receptors on detrusor cholinergic nerve terminals indirectly mediating the inhibitory role of β_3-adrenoceptor agonists in the bladder of anaesthetized rats has been demonstrated before (Silva et al., 2017). This prompted us to investigate the intracellular mechanism downstream β_3-adrenoceptor-induced AC activation and cAMP accumulation in the urinary bladder of anaesthetized rats. Figure 4 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 FSK (0.01–10 μM, Figure 4b$_i$) and isoprenaline (0.1–1,000 nM, Figure 4b$_{ii}$) into the bladder lumen, whereas inhibition of PKA with H-89 (10 μM) had no significant effect. None of these drugs affected the urodynamic parameters when applied alone (Figure 4a). Instillation of H-89 (10 μM) and ESI-09 (10 μM) into the bladder lumen also did not significantly (P > .05) affect the heart rate (429 ± 28 bpm, n = 12 and 416 ± 62 bpm, n = 11, respectively) in urethane-anaesthetized rats when compared to the saline infusion (425 ± 28 bpm, n = 12 and 392 ± 69 bpm, n = 11, respectively).

3.5 | β_3-adrenoceptor inhibition of the rat urinary bladder function involves EPAC1 and downstream PKC activation resulting in adenosine overflow via ENT1

Data from the literature suggest that EPAC may serve as an intermediate mediator linking the β-adrenoceptor–cAMP pathway to **PKC** activity. Evidence comes from the observation that β-adrenoceptors activation induces PKC translocation to the plasma membrane, an effect that was independent of PKA activation but was mimicked by selective EPAC agonists (Hucho, Dina, & Levine, 2005). β-adrenoceptor-induced translocation of PKC was abrogated by inhibiting EPAC expression in cardiomyocytes from newborn rats (Duquesnes et al., 2010; Li, Cai, Liu, & Guo, 2015). In addition, U73122, an inhibitor of **PLC** activity, attenuated PKC translocation after activation of β-adrenoceptors (Hucho et al., 2005) and EPAC (Li et al., 2015). This prompted us to investigate the contribution of PLC and PKC to β_3-adrenoceptors-induced inhibition of the rat urinary bladder function.

Figure 4b$_{ii}$ shows that the PKC inhibitor, chelerythrine (5 μM), prevented the inhibitory role of isoprenaline (0.1–1,000 nM) on the voiding frequency to a similar extent to that caused by the EPAC inhibitor, ESI-09 (10 μM). On its own, intravesical chelerythrine (5 μM) did not significantly (P > .05) affect other urodynamic parameters neither the heart rate of urethane-anaesthetized rats.

Selective activation of (a) EPAC1 with the cAMP analogue, 8-CPT-2Me-cAMP (20 μM, Figure 5a) and of (b) PKC with PMA (10 μM, Figure 5b) mimicked the inhibitory effects of mirabegron (0.1 μM; Figure 3b), isoprenaline (1 μM; Figure 3c), and FSK (3 μM; Figure 3d) on electrically evoked [^{3}H]ACh release from urothelium-denuded human and rat detrusor strips.

The inhibitory effects of 8-CPT-2Me-cAMP (20 μM) and PMA (10 μM) were prevented by ESI-09 (10 μM, Figure 5a) and chelerythrine (5 μM, Figure 5b) in rat detrusor strips, thus indicating the involvement of EPAC1 and PKC, respectively. Involvement of adenosine outflow via the ENT1 transporter system was confirmed because Dipy (5 μM) prevented inhibition of [^{3}H]ACh release from rat detrusor strips caused by both 8-CPT-2Me-cAMP (20 μM, Figure 5a) and PMA (10 μM, Figure 5b).

In view of the putative involvement of the PLC–PKC pathway mediating intracellular EPAC signals in different tissues, we also

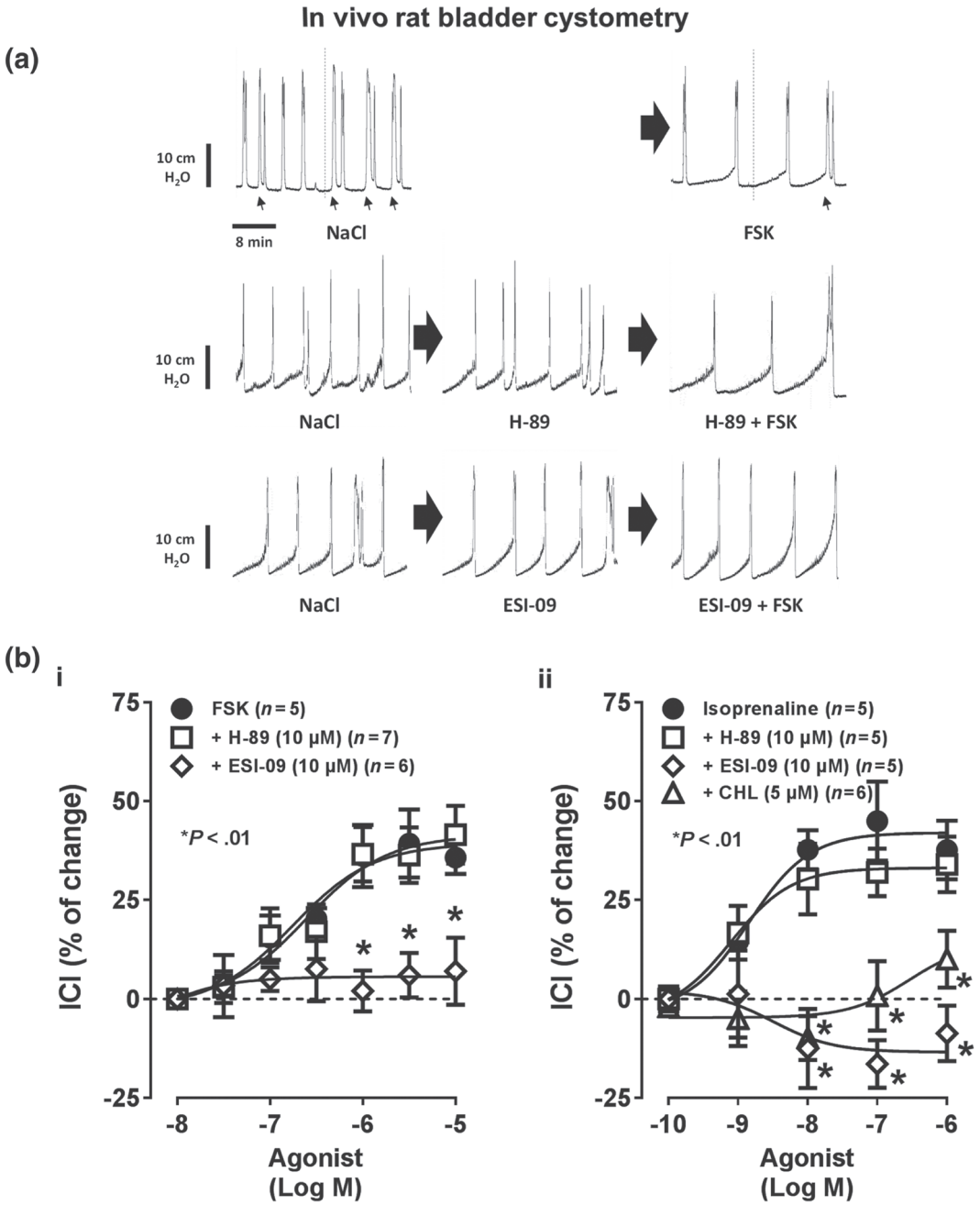

FIGURE 4 (a) Bladder cystometry recordings during normal saline (0.9% w/v of NaCl) and forskolin (FSK, 3 μM) infusion into the urinary bladder of urethane-anaesthetized rats. Large-amplitude bladder contractions correspond to voiding contractions when they were accompanied by urine draining through the urethra. Black arrows represent non-voiding bladder contractions that were not followed by urine drainage through the urethra; its number decreased in the presence of FSK (3 μM). Stable urodynamic responses to FSK were reached in 10–15 min. The effect of FSK (3 μM) after inhibition of PKA with H-89 (10 nM) and of EPAC with ESI-09 (10 μM) is also shown for comparison. (b) Show concentration–response curves of FSK (b_i, 0.01–10 μM)- and isoprenaline (b_{ii}, 0.1–1,000 nM)-induced prolongation of the intercontraction interval (ICI) of voiding contractions in the absence and in presence of inhibitors of PKA (H-89, 10 nM), EPAC (ESI-09, 10 μM), and PKC (CHL, 5 μM). Control values correspond to zero percent variation. The vertical bars represent SD of an *n* number of animals (shown in parentheses). $^*P < .05$ (two-way ANOVA followed by the Dunnett's multiple comparison test) represents significant differences as compared to the effect of FSK (b_i) or isoprenaline (b_{ii}) applied alone

investigated the release inhibitory action of isoprenaline (1 μM) in the presence of PLC and PKC inhibitors. Inhibition of PKC with chelerythrine (5 μM) and Go6976 (30 nM, a selective inhibitor of Ca^{2+}-dependent PKCα and PKCβI), but not of PLC with U73122 (3 μM), significantly ($P < .05$) attenuated isoprenaline (1 μM)-induced inhibition of evoked [^{3}H]ACh release from urothelium-denuded rat detrusor strips (Figure 6a). Likewise, chelerythrine (5 μM) also attenuated the inhibitory effect of mirabegron (0.1 μM) on evoked transmitter release

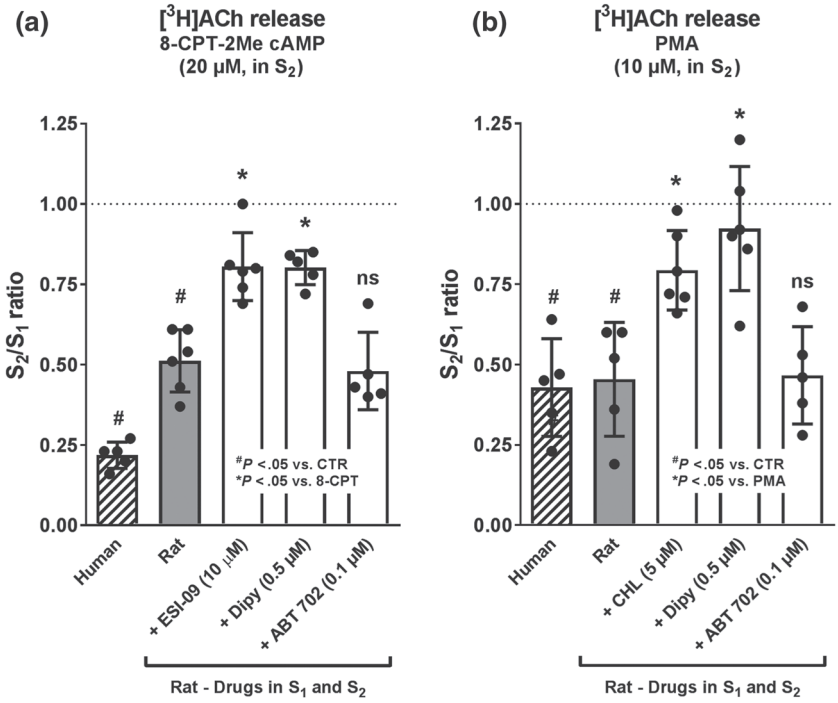

FIGURE 5 Activation of EPAC (with 8-CPT-2Me cAMP, a) and PKC (with phorbol 12-myristate 13-acetate, PMA, b) decrease electrical evoked (10 Hz, 200 pulses of 0.2-ms duration) [^{3}H]ACh release from urothelium-denuded human and rat detrusor strips. In rat detrusor strips, 8-CPT-2Me cAMP (20 μM) and PMA (10 μM) were applied 6 min before S_2 either in the absence or in the presence of dipyridamole (Dipy, 0.5 μM) and ABT 702 (0.1 μM) to inhibit ENT1 and adenosine kinase, respectively; ESI-09 (10 μM) and chelerythrine (CHL, 5 μM) were also used to show that the inhibitory effects of CPT-2Me cAMP (20 μM) and PMA (10 μM) were due to EPAC and PKC activation respectively. All inhibitors 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 an *n* number of individuals (black dots). $^{\#,*}P < .05$ (one-way ANOVA followed by the Dunnett's multicomparison test with a single pooled variance) represent significant differences when compared to the control situation and to the inhibitory effects of 8-CPT-2Me cAMP (a) and PMA (b) applied alone to rat detrusor strips respectively

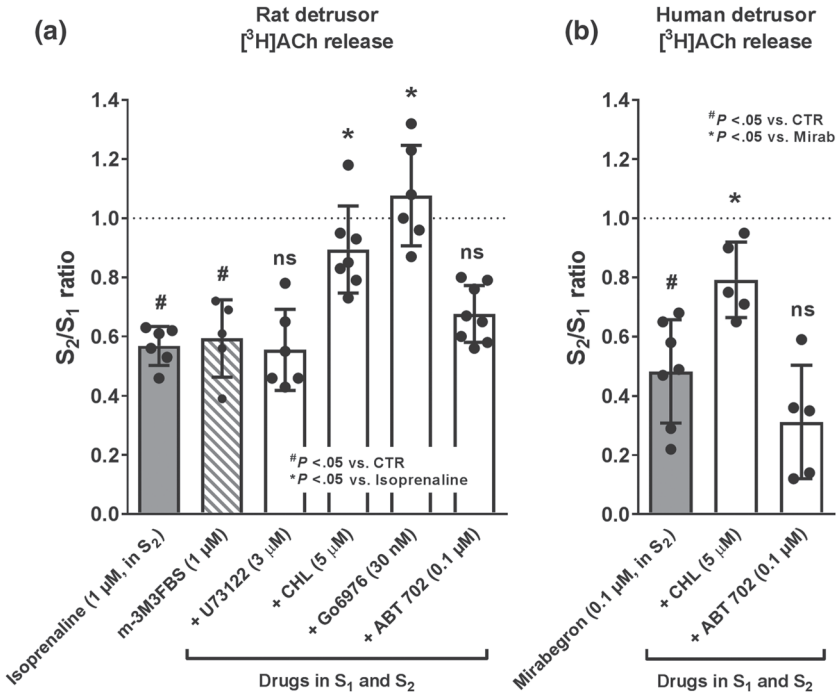

FIGURE 6 Influence of the PKC pathway on the inhibitory effects of isoprenaline and mirabegron on electrically evoked (10 Hz, 200 pulses of 0.2-ms duration) [^{3}H]ACh release from urothelium-denuded rat (a) and human (b) detrusor strips. The effect of the PLC activator, m-3-M3-FBS (3 μM), is shown for comparison. Isoprenaline (1 μM) and mirabegron (0.1 μM) were applied 6 min before S_2 either in the absence or in the presence of the PLC inhibitor, U73122 (3 μM), and of two PKC inhibitors, chelerythrine (CHL, 5 μM) and Go6976 (30 nM); the putative effect of PKC-induced changes in adenosine kinase (ADK) activity triggered by β_3-adrenoceptors stimulation was tested using the ADK inhibitor, ABT 702 (0.1 μM). All inhibitors 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 an *n* number of individuals (black dots). $^{\#,*}P < .05$ (one-way ANOVA followed by the Dunnett's multicomparison test with a single pooled variance) represent significant differences when compared to the control situation or to the inhibitory effects of isoprenaline (a) and mirabegron (b) applied alone respectively

from urothelium-denuded human detrusor strips (Figure 6b). In parallel to the effect of chelerythrine (5 μM) on β_3-adrenoceptor-induced inhibition of transmitter exocytosis, the PKC inhibitor abolished adenosine release from rat detrusor strips stimulated with isoprenaline (1 μM) under resting conditions, that is, isoprenaline (1 μM) increased extracellular adenosine from 9 ± 1 to 38 ± 3 pmol·mg^{-1} in

control conditions (n = 6, P < .05) but only from 15 ± 2 to 19 ± 2 pmol·mg^{-1} in the presence of chelerythrine (5 μM; n = 5, P > .05).

Overall, data suggest that β_3-adrenoceptor-induced inhibition of nerve-evoked [^{3}H]ACh release in the rat detrusor indirectly depends on extracellular adenosine accumulation through a mechanism involving EPAC1 and downstream PKC stimulation without needing the activation of membrane-associated PLC. This does not necessarily mean that activation of PLC by any other mechanism resulting in PKC stimulation is unable to reduce transmitter release from stimulated cholinergic bladder nerves. To test this hypothesis and to rule out questions about the specificity of U73122 to inhibit PLC (Burgdorf, Schäfer, Richardt, & Kurz, 2010; Leitner et al., 2016), we used the PLC activator, m-3M3FBS (1 μM), as a positive control. On its own, m-3M3FBS (1 μM) decreased nerve-evoked [^{3}H]ACh release from the rat detrusor by a similar amount (40 ± 5%, n = 5) to that caused by isoprenaline (1 μM; 39 ± 1%, n = 6).

PKC activation may favour adenosine outflow via ENT1 (Coe, Zhang, McKenzie, & Naydenova, 2002) through a mechanism involving stimulation of 5′-nucleotidase (Obata, Kubota, & Yamanaka, 2001) and/or inhibition of adenosine kinase (ADK; Sinclair, Shepel, Geiger, & Parkinson, 2000) resulting in the intracellular accumulation of the nucleoside and its translocation to the extracellular milieu via ENTs. Figure 6 shows that selective inhibition of ADK with the non-nucleoside drug, ABT 702 (0.1 μM), did not modify the inhibitory effects of isoprenaline (1 μM; Figure 6a) and mirabegron (0.1 μM; Figure 6b) on evoked [^{3}H]ACh release from rat and human detrusor strips respectively. ABT 702 (0.1 μM) also failed to affect transmitter release inhibition caused by 8-CPT-2Me-cAMP (20 μM; Figure 5a) and PMA (10 μM; Figure 5b) in the rat detrusor. Despite that on its own, the ADK inhibitor significantly (P < .05) reduced the evoked [^{3}H]ACh release from both rat (S_2/S_1 ratio 0.41 ± 0.04, n = 6) and human (S_2/S_1 ratio 0.29 ± 0.06, n = 5) detrusor strips (data not shown), data suggest that PKC-induced ADK inhibition does not seem to play a role on β_3-adrenoceptors-mediated down-regulation of cholinergic neurotransmission in the rat urinary bladder.

4 | DISCUSSION AND CONCLUSIONS

In a previous study from our group, we showed that inhibition of cholinergic neurotransmission by β_3_adrenoceptors activation is indirectly mediated by adenosine released from detrusor smooth muscle fibres, via ENT1, leading to retrograde activation of inhibitory A_1 receptors on cholinergic nerves of human and rat urinary bladders (Silva et al., 2017). While A_1 receptors are predominantly localized in vesicular ACh transporter-positive cholinergic nerve terminals (Silva et al., 2017; Silva-Ramos et al., 2015), we and others showed that detrusor smooth muscle fibres possess the highest β_3-adrenoceptors density in the urinary bladder of humans, rats and mice. This was determined by immunofluorescence confocal microscopy and Western blot analysis using multiple antibodies targeting different receptor epitopes (Griffin et al., 2018; Igawa et al., 1999; Silva et al., 2017). Detection of β_3-adrenoceptors on ACh-containing nerve fibres of the human bladder is controversial (Coelho, Antunes-Lopes, Gillespie, & Cruz, 2017; but see Otsuka et al., 2013; Silva et al., 2017). This is a yet unresolved issue mostly because such studies have (a) deficiencies in immunostaining validation criteria recommending the use of antibodies against multiple β_3-adrenoceptor epitopes (Cernecka et al., 2012; Silva et al., 2017) and (b) technical differences in tissue fixation, slice thickness and documentation using low resolution epifluorescence microscopy without autofluorescence subtraction (discussed in Okeke et al., 2017; Silva et al., 2017). Notwithstanding this, data also suggests that β_3_adrenoceptor agonists may decrease bladder overactivity by fine-tuning regulating by the sensory bladder drive during urine storage through adenosine release from mechanically stimulated urothelium cells in anaesthetized rats. The close proximity of β_3_adrenoceptors and adenosine A_1 receptors in umbrella cells and the presence of ENT1 transporters in the basolateral membrane of these cells directly implicates adenosine as a main inhibitory controller of bladder function. Here, we provide additional evidence showing that β_3-adrenoceptor-induced inhibition of bladder activity involves a preferential stimulation of the EPAC1–PKC pathway downstream cAMP, which ultimately results in ENT1-mediated translocation of adenosine to the extracellular milieu in human and rat urinary bladders (Figure 7).

β_3_Adrenoceptors usually couple to AC via $G_{\alpha s}$ proteins leading to intracellular cAMP accumulation (Tyagi, Thomas, Yoshimura, & Chancellor, 2009). Both cAMP-dependent and -independent pathways may be involved in relaxation of the detrusor (Maki et al., 2019; Uchida, Shishido, Nomiya, & Yamaguchi, 2005; see Michel & Vrydag, 2006; Yamaguchi & Chapple, 2007), while stimulation of these pathways normally facilitate transmitter release from stimulated cholinergic nerve terminals (see, e.g., Correia-de-Sá & Ribeiro, 1994; Correia-de-Sá, Timóteo, & Ribeiro, 2000; Oliveira & Correia-de-Sá, 2005; Vieira et al., 2009). Here, we show that β_3_adrenoceptors agonists (e.g. mirabegron and isoprenaline) and the AC catalytic subunit activator (FSK) reduce by a similar amount [^{3}H]ACh release from stimulated human and rat detrusor strips without the mucosa. These compounds also diminished the micturition frequency, suggesting that the urinary bladder compliance depends on cAMP production downstream of β_3_adrenoceptors activation.

Intracellular cAMP can be rapidly converted into AMP and adenosine by highly effective PDEs and 5′-nucleotidase respectively. Thus, cAMP accumulation indirectly raises the transmembrane gradient of adenosine forcing the nucleoside outflow to the extracellular milieu. Using immunofluorescence confocal imaging and Western blot analysis to detect the presence of β_3-adrenoceptors and ENT1 transporters in the plasma membrane of detrusor smooth muscle fibres in combination with HPLC to quantify adenosine in the extracellular fluid, we proved that β_3-adrenoceptors activation endorses adenosine release from non-stimulated detrusor strips via NBTI- and Dipy-sensitive ENT1 transporters, both in humans and in rats. Besides contributing to generate adenosine, β_3-adrenoceptors-induced cAMP may activate downstream intracellular signalling pathways. The cellular effects of cAMP are most often attributed to PKA (reviewed in Wang et al., 2017). However, detrusor relaxation owing to AC/PKA pathway

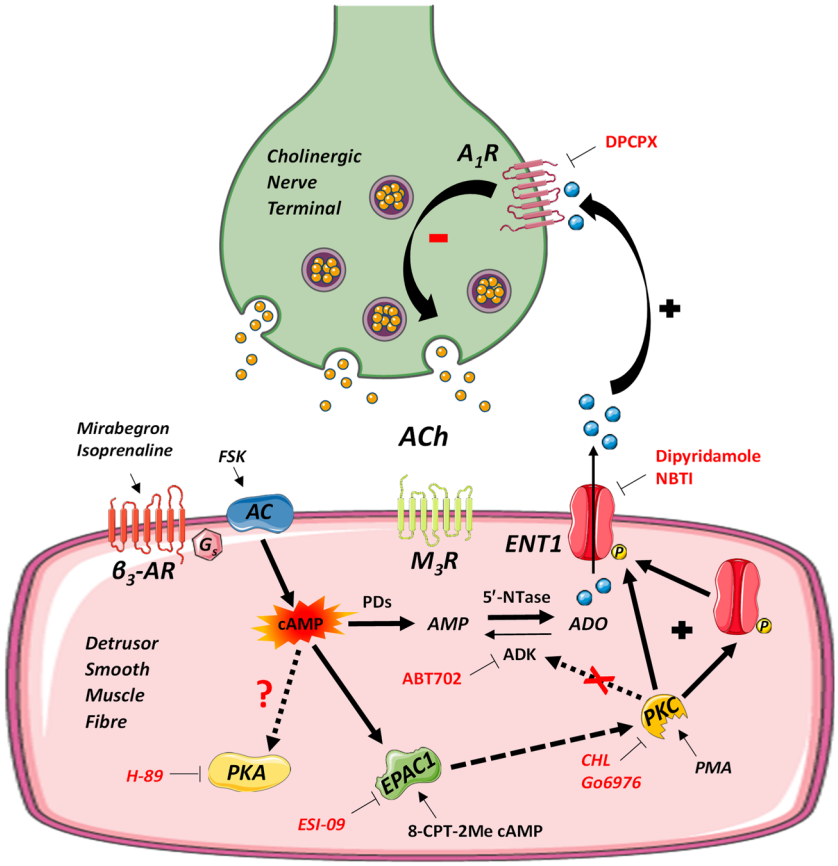

FIGURE 7 Schematic representation of the mechanisms involved on β_3-adrenoceptors inhibition of cholinergic neurotransmission in human and rat urinary bladders. β_3-adrenoceptors (β_3-AR) predominantly located in detrusor smooth fibres are positively coupled to AC leading to increases in intracellular cAMP accumulation. The enzymatic breakdown of cAMP by intracellular PDEs and 5′-nucleotidase (5′NTase) results in the formation of high levels of adenosine (ADO) forcing its translocation to the extracellular milieu, via dipyridamole-sensitive ENT1 transporters. Besides acting as a source of ADO, cAMP triggers an intracellular signalling cascade leading to preferential EPAC1 over PKA activation on detrusor smooth muscle fibres. Data suggest that EPAC-induced activation of calcium-dependent conventional PKC isoforms may be necessary to stimulate ENT1-mediated ADO outflow. The way PKC facilitates ADO transport to the extracellular compartment is still unknown, but it may result from PKC phosphorylation-induced increases in the nucleoside transporters capacity and/or by favouring their translocation to the plasma membrane. Once in the extracellular milieu, ADO is free to activate inhibitory A_1 receptors (A_1R) on cholinergic nerve terminals causing the inhibition of nerve-evoked ACh release

downstream activation of β_3-adrenoceptors has been questioned (Frazier et al., 2005; Maki et al., 2019), raising the hypothesis that PKA-independent effects may be involved.

Mounting evidence point towards novel cAMP targets to explain cell responses that are insensitive to PKA inhibition (see Dekkers et al., 2013); these include the EPAC (de Rooij et al., 1998; Kawasaki et al., 1998). Unlike PKA, EPAC is a cAMP-regulated guanine nucleotide exchange factor, with no kinase activity, that favours GDP/GTP exchange and, thereby, activation of small Ras-like GTPases, such as Rho, Rac and Ras (de Rooij et al., 1998; Kawasaki et al., 1998). The expression of EPAC1, but not EPAC2 was detected by Western blot analysis alongside EPAC downstream targets Rac1 and Rap1 in the human detrusor smooth muscle (Hayashi et al., 2016). Here, we confirmed using immunofluorescence confocal microscopy and Western blot analysis that EPAC1 is the dominant isoform in the urothelium-denuded human and rat detrusor. While EPAC did not seem to play a significant role in modulating the activity of the contractile machinery in α-toxin permeabilized human detrusor challenged with the muscarinic agonist, carbachol (Hayashi et al., 2016), here we raise a novel hypothesis suggesting that the EPAC signalling pathway is a major contributor to the inhibitory effect of β_3-adrenoceptors on cholinergic neurotransmission in human and rat urinary bladders. The participation of the EPAC signalling cascade in cAMP-related cell adhesion, cell communication, vesicle trafficking and secretion, as well as in fibrocytes viability, proliferation and differentiation (Certal et al., 2015), 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.

Our results show that the EPAC inhibitor, ESI-09, mimicked the preventive role of NBTI and Dipy on adenosine release caused by both mirabegron and isoprenaline in human and rat detrusor strips, respectively, but the effect of ESI-09 was not shared by the selective EPAC2 inhibitor, ESI-05. Thus, inhibition of EPAC1, but not of EPAC2 nor PKA, removed the β_3-adrenoceptor inhibitory control of [^{3}H]ACh release from stimulated detrusor strips. A similar inhibitory interaction was observed when the AC activator (FSK) was used instead of β_3-adrenoceptor agonists. Co-localization of β_3-adrenoceptors, EPAC1 and ENT1 transporters in human and rat detrusor smooth muscle fibres (this study; cf. Hayashi et al., 2016) consolidate our theory that EPAC1 mediates β_3-adrenoceptor-induced cholinergic inhibition via the release of adenosine through ENT1 transporters. The proximity of all these players in human (Otsuka, Shinbo, Matsumoto, Kurita, & Ozono, 2008; Silva et al., 2017) and rat (Nakagomi et al., 2015) urothelial cells may also contribute to explain why the negative influence of isoprenaline and FSK in the voiding frequency was inhibited by the EPAC inhibitor in a similar proportion to that obtained with ENT1 blockers, Dipy and NBTI (Silva et al., 2017).

As for the signalling cascades involving EPAC, it is known that deletion of EPAC1 inibited β-adrenoceptor-induced translocation of PKC to the plasma membrane in rat cardiomyocyte cells, thus

suggesting that the EPAC1 signalling is upstream of PKC translocation upon β-adrenoceptor activation (Chen et al., 2012). These findings suggest that EPAC1 may be the link between β-adrenoceptors activation and the PLC–PKC transduction pathway. PKC activation exerts a predominant inhibitory action by controlling spontaneous non-voiding contractions during the urine filling phase to increase bladder storage capacity and to delay the need to void (Hypolite, Chang, Wein, Chacko, & Malykhina, 2015). The same was observed with β_3-adrenoceptor agonists (Hatanaka et al., 2013; Silva et al., 2017). Our data show that PKC inhibition prevented the inhibitory action of isoprenaline on transmitter release from isolated detrusor strips and increased bladder reactivity in anaesthetized rats. The effect of chelerythrine mimicked that obtained by inhibiting EPAC1, adenosine outflow via ENT1 and presynaptic A_1 receptors with ESI-09, NBTI/Dipy, and DPCPX respectively. Blocking PKC with chelerythrine also attenuated the inhibitory effect of mirabegron on evoked [^{3}H]ACh release from human detrusor strips. Downstream stimulation of PKC by EPAC1 without requiring membrane-associated PLC activation is supported by the fact that PMA, a widely used PKC activator, mimicked the inhibitory effects of β_3-adrenoceptor agonists, as well as of activators of AC (FSK) and EPAC1 (8-CPT-2Me-cAMP), on cholinergic neurotransmission in human and rat urinary bladders. Tandem activation of EPAC1 and PKC results in adenosine-mediated effects, since the inhibitory actions of 8-CPT-2Me-cAMP and PMA were equally prevented by the ENT1 inhibitor, Dipy on evoked [^{3}H]ACh release from rat detrusor strips.

Both Ca^{2+}-dependent (Gu, Li, Chen, & Huang, 2016) and Ca^{2+}-independent (Li et al., 2015; Lipp & Reither, 2011) isoforms of PKC may act as downstream effectors of EPAC activation. Our data show that the preventing effect of chelerythrine on isoprenaline-induced inhibition of evoked [^{3}H]ACh release was mimicked by Go6976, a selective inhibitor of Ca^{2+}-dependent conventional PKCα and PKCβI isoforms. It remains, however, to elucidate how EPAC can activate PKC, since a direct interaction between these two proteins has not been reported yet. Intracellular Ca^{2+} accumulation may be putatively involved in EPAC–PKC interaction given to the fact that EPAC can trigger Ca^{2+} mobilization from smooth muscle cells sarcoplasmic reticulum (Holz, Kang, Harbeck, Roe, & Chepurny, 2006). Most often, EPAC-dependent Ca^{2+} mobilization requires PLC activation via Rap1 (Oestreich et al., 2007), a situation that does not seem to occur under the present experimental conditions since inhibition of PLC with U73122 did not change the inhibitory action of isoprenaline on cholinergic neurotransmission in the rat bladder, despite the fact that the PLC activator, m-3M3FBS, decreased nerve-evoked [^{3}H]ACh release from the rat detrusor by a similar amount to that caused by isoprenaline. Further studies are required to explore other putative mechanisms, including a direct interaction of EPAC1 with sarcoplasmic pathways controlling Ca^{2+} release and/or an indirect effect mediated via the Rap–ERK pathway (Holz et al., 2006).

The mechanism by which the EPAC1–PKC pathway regulates ENT1-mediated adenosine release was not directly addressed in this study. Nevertheless, both human and mouse ENT1 are directly phosphorylated by both PKC and PKA (Reyes et al., 2011). Thus, post-translational modification of ENT1 by PKC-dependent phosphorylation can potentially serve as a mechanism to control transport efficacy (Coe et al., 2002; Fernández-Calotti et al., 2008). PKC-operated regulation of substrate transport can be achieved by several distinct mechanisms, including modulation of the transporter expression, either at a transcriptional (mRNA) or translational (protein) level. Kinases can also regulate the transporter localization by promoting its translocation to the plasma membrane or by facilitating its internalization. Transporter intrinsic activity may also be affected by kinase-mediated phosphorylation (Mayati et al., 2017). The mechanism by which PKC activity controls ENT1 function is still an open question, with some authors arguing that PKC alters the active state of already existing ENT1 at the plasma membrane (Coe et al., 2002; Fernández-Calotti et al., 2008), while other authors suggest that phosphorylation of serine 281 residue increases human ENT1 density at the plasma membrane possibly by increasing its translocation from intracellular stores (Hughes, Cravetchi, Vilas, & Hammond, 2015; see Figure 7). Another possibility to foster adenosine outflow via ENT1 is by increasing the intracellular concentration of the nucleoside through PKC-mediated inhibition of ADK (Sinclair et al., 2000). However, this theory was not proven under the present experimental conditions since the ADK inhibitor, ABT 702, failed to modify the effects of β_3-adrenoceptor agonists, as well as of the activators of EPAC1 and PKC, on evoked [^{3}H]ACh release from human and rat detrusor strips.

In conclusion, data suggest that the therapeutic success of β_3-adrenoceptor agonists in the treatment of OAB syndromes involves activation of an EPAC1–PKC intracellular pathway downstream of cAMP production, resulting in adenosine release, via ENT1, from the detrusor and retrograde activation of inhibitory A_1 receptors on cholinergic nerves. The adenosine-mediated inhibitory effect on cholinergic neurotransmission (this study; Silva et al., 2017) may add to cAMP-dependent and -independent mechanisms whenever β_3-adrenoceptor agonists reach the micromolar concentration range required to cause direct relaxation of detrusor smooth muscle fibres (Maki et al., 2019; Michel & Korstanje, 2016). Although still premature, manipulation of the activity of EPAC1 and conventional PKC isoforms may provide additional therapeutic targets to improve bladder overactivity, alongside β_3-adrenoceptor agonists, also counting on the possibility that the cAMP-dependent EPAC1 signalling may prevent fibroblast-induced irreversible bladder remodelling that is often observed as a consequence of bladder obstruction and/or chronic overactivation (see Certal et al., 2015).

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AUTHOR CONTRIBUTIONS

P.C.S. supervised the project, designed the experiments, and analysed all data. I.S. and P.C.S. wrote the paper. I.S., D.S., C.V., and T.M.C. performed the experiments and quantified adenosine release by HPLC. I.S. and C.V. performed ACh release experiments and analysed the data. A.F.C., S.M., and D.S. performed in vivo cystometry experiments and analysed the data. I.S., D.S., C.V., and F.F. performed confocal microscopy experiments and Western blot analysis. M.S.R. recruited organ donors and collected bladder samples. I.S., T.M.C., F.F., S.M., A.F.C., D.S., C.V., M.S.R., and P.C.S. interpreted the data, discussed the clinical implications, and commented on the manuscript at all stages.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the BJP guidelines for **Design & Analysis**, **Immunoblotting and Immunochemistry** and **Animal Experimentation**, and as recommended by funding agencies, publishers and other organisations engaged with supporting research.

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REFERENCES

Aizawa, N., Homma, Y., & Igawa, Y. (2015). Effects of L-arginine, mirabegron and oxybutynin on the primary afferent nerve activities synchronized with reflexic, rhythmic bladder contractions in the rat. *Neurourology and Urodynamics*, *34*, 368–374.

Alexander, S. P. H., Christopoulos, A., Davenport, A. P., Kelly, E., Mathie, A., Peter, J. A., … CGTP Collaborators (2019). The concise guide to pharmacology 2019/2020: G protein-coupled receptors. *British Journal of Pharmacology*, *176*, S21–S141.

Alexander, S. P., Fabbro, D., Kelly, E., Mathie, A., Peters, J. A., Veale, E. L., … CGTP collaborators (2019). The concise guide to pharmacology 2019/20: Enzymes. *British Journal of Pharmacology*, *176*, S297–S396.

Alexander, S. P. H., Kelly, E., Mathie, A., Peters, J. A., Veale, E. L., Armstrong, J. F., … Collaborators, GTP (2019). The concise guide to pharmacology 2019/20: Transporters. *British Journal of Pharmacology*, *176*, 397–497.

Alexander, S. P. H., Roberts, R. E., Broughton, B. R. S., Sobey, C. G., George, C. H., Stanford, S. C., … Ahluwalia, A. (2018). Goals and practicalities of immunoblotting and immunohistochemistry: A guide for submission to the British Journal of Pharmacology. *British Journal of Pharmacology*, *175*, 407–411.

Almahariq, M., Chao, C., Mei, F. C., Hellmich, M. R., Patrikeev, I., Motamedi, M., & Cheng, X. (2014). Pharmacological inhibition and genetic knockdown of exchange protein directly activated by cAMP 1 reduce pancreatic cancer metastasis in vivo. *Molecular Pharmacology*, *87*, 142–149.

Almahariq, M., Tsalkova, T., Mei, F. C., Chen, H., Zhou, J., Sastry, S. K., … Cheng, X. (2013). A novel EPAC-specific inhibitor suppresses pancreatic cancer cell migration and invasion. *Molecular Pharmacology*, *83*, 122–128. https://doi.org/10.1124/mol.112.080689

Andersson, K. E. (2017). On the site and mechanism of action of β_3-adrenoceptor agonists in the bladder. *International Neurourology Journal*, *21*, 6–11.

Angulo, J. C., Khullar, V., Nitti, V. W., & Siddiqui, E. (2013). Evidence available on the use of the selective β_3-adrenoceptor agonist mirabegron for the treatment of overactive bladder. *Actas Urologicas Españolas*, *37*, 640–651.

Baker, J. G. (2010). The selectivity of β-adrenoceptor agonists at human β1-, β2- and β3-adrenoceptors. *British Journal of Pharmacology*, *160*, 1048–1061.

Barros-Barbosa, A. R., Ferreirinha, F., Oliveira, Â., Mendes, M., Lobo, M. G., Santos, A., … Correia-de-Sá, P. (2016). Adenosine A_{2A} receptor and ecto-5′-nucleotidase/CD73 are upregulated in hippocampal astrocytes of human patients with mesial temporal lobe epilepsy (MTLE). *Purinergic Signal*, *12*, 719–734.

Bell, D., & McDermott, B. J. (1994). Use of the cyclic AMP antagonist, Rp-cAMPS, to distinguish between cyclic AMP-dependent and cyclic AMP-independent contractile responses in rat ventricular cardiomyocytes. *Journal of Molecular and Cellular Cardiology*, *26*, 1439–1448.

Burgdorf, C., Schäfer, U., Richardt, G., & Kurz, T. (2010). U73122, an aminosteroid phospholipase C inhibitor, is a potent inhibitor of cardiac phospholipase D by a PIP2-dependent mechanism. *Journal of Cardiovascular Pharmacology*, *55*, 555–559. https://doi.org/10.1097/FJC.0b013e3181d8bec5

Candelore, M. R., Deng, L., Tota, L., Guan, X. M., Amend, A., Liu, Y., … Weber, A. E. (1999). Potent and selective human β3-adrenergic receptor antagonists. *The Journal of Pharmacology and Experimental Therapeutics*, *290*, 649–655.

Carneiro, I., Timóteo, M. A., Silva, I., Vieira, C., Baldaia, C., Ferreirinha, F. … Correia-de-Sá, P. (2014). Activation of $P2Y_6$ receptors increases the voiding frequency in anaesthetized rats by releasing ATP from the bladder urothelium. *British Journal of Pharmacology*, *174*, 3404–3419.

Cernecka, H., Ochodnicky, P., Lamers, W. H., & Michel, M. C. (2012). Specificity evaluation of antibodies against human β_3-adrenoceptors. *Naunyn-Schmiedeberg's Archives of Pharmacology*, *385*, 875–882.

Cernecka, H., Sand, C., & Michel, M. C. (2014). The odd sibling: Features of β_3-adrenoceptor pharmacology. *Molecular Pharmacology*, *86*, 479–484.

Certal, M., Vinhas, A., Pinheiro, A. R., Ferreirinha, F., Barros-Barbosa, A. R., Silva, I., … Correia-de-Sá, P. (2015). Calcium signaling and the novel anti-proliferative effect of the UTP-sensitive $P2Y_{11}$ receptor in rat cardiac myofibroblasts. *Cell Calcium*, *58*, 518–533.

Chen, C., Du, J., Feng, W., Song, Y., Lu, Z., Xu, M., … Zhang, Y. (2012). β-Adrenergic receptors stimulate interleukin-6 production through Epac-dependent activation of PKCIδ/p38 MAPK signalling in neonatal mouse cardiac fibroblasts. *British Journal of Pharmacology*, *166*, 676–688.

Cheng, X., Ji, Z., Tsalkova, T., & Mei, F. (2008). Epac and PKA: A tale of two intracellular cAMP receptors. *Acta Biochimica et Biophysica Sinica Shanghai*, *40*, 651–662. https://doi.org/10.1111/j.1745-7270.2008.00438.x

Coe, I., Zhang, Y., McKenzie, T., & Naydenova, Z. (2002). PKC regulation of the human equilibrative nucleoside transporter, hENT1. *FEBS Letters*, *517*, 201–205.

Coelho, A., Antunes-Lopes, T., Gillespie, J., & Cruz, F. (2017). β-3 adrenergic receptor is expressed in acetylcholine-containing nerve fibers of the human urinary bladder: An immunohistochemical study. *Neurourology and Urodynamics*, *36*, 1972–1980.

Correia-de-Sá, P., & Ribeiro, J. A. (1994). Evidence that the presynaptic A_{2a}-adenosine receptor of the rat motor nerve endings is positively coupled to adenylate cyclase. *Naunyn-Schmiedeberg's Archives of Pharmacology*, *350*, 514–522.

Correia-de-Sá, P., Timóteo, M. A., & Ribeiro, J. A. (2000). Influence of stimulation on Ca^{2+} recruitment triggering [^{3}H]acetylcholine release from the rat motor-nerve endings. *European Journal of Pharmacology*, *406*, 355–362. https://doi.org/10.1016/s0014-2999(00)00686-5

Curtis, M. J., Ashton, J. C., Moon, L. D. F., & Ahluwalia, A. (2018). Clarification of the basis for the selection of requirements for publication in the British Journal of Pharmacology. *British Journal of Pharmacology*, *175*, 3633–3635. https://doi.org/10.1111/bph.14443

D'Agostino, G., Condino, A. M., & Calvi, P. (2015). 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. *European Journal of Pharmacology*, *758*, 115–122.

Dekkers, B. G., Racké, K., & Schmidt, M. (2013). Distinct PKA and EPAC compartmentalization in airway function and plasticity. *Pharmacology & Therapeutics*, *137*, 248–265.

de Rooij, J., Zwartkruis, F. J., Verheijen, M. H., Coll, R. H., Nijman, S. M., Wittinghofer, A., … Bos, J. L. (1998). Epac is a Rap1 guanine-nucleotide-exchange factor directly activated by cyclic AMP. *Nature*, *396*, 474–477. https://doi.org/10.1038/24884

Duquesnes, N., Derangeon, M., Métrich, M., Lucas, A., Mateo, P., Li, L., … Crozatier, B. (2010). Epac stimulation induces rapid increases in connexin43 phosphorylation and function without preconditioning effect. *Pflügers Archiv - European Journal of Physiology*, *460*, 731–741.

Evans, B. A., Papaioannou, M., Hamilton, S., & Summers, R. J. (1999). Alternative splicing generates two isoforms of the β3-adrenoceptor which are differentially expressed in mouse tissues. *British Journal of Pharmacology*, *127*, 1525–1531.

Fernández-Calotti, P., Galmarini, C. M., Cañones, C., Gamberale, R., Saénz, D., Avalos, J. S., … Giordano, M. (2008). Modulation of the human equilibrative nucleoside transporter1 (hENT1) activity by IL-4 and PMA in B cells from chronic lymphocytic leukemia. *Biochemical Pharmacology*, *75*, 857–865.

Frazier, E. P., Mathy, M. J., Peters, S. L., & Michel, M. C. (2005). Does cyclic AMP mediate rat urinary bladder relaxation by isoproterenol? *The Journal of Pharmacology and Experimental Therapeutics*, *313*, 260–267.

Gong, B., Shelite, T., Mei, F. C., Ha, T., Hu, Y., Xu, G., … Cheng, X. (2013). Exchange protein directly activated by cAMP plays a critical role in bacterial invasion during fatal rickettsioses. *Proceedings of the National Academy of Sciences*, *110*, 19615–19620.

Griffin, C. S., Bradley, E., Hollywood, M. A., McHale, N. G., Thornbury, K. D., & Sergeant, G. P. (2018). β_3-adrenoceptor agonists inhibit carbachol-evoked Ca^{2+} oscillations in murine detrusor myocytes. *BJU International*, *121*, 959–970. https://doi.org/10.1111/bju.14090

Gu, Y., Li, G., Chen, Y., & Huang, L.-Y. M. (2016). Epac–protein kinase C α signaling in purinergic P2X3R-mediated hyperalgesia after inflammation. *Pain*, *157*, 1541–1550. https://doi.org/10.1097/j.pain.0000000000000547

Harding, S. D., Sharman, J. L., Faccenda, E., Southan, C., Pawson, A. J., Ireland, S., … NC-IUPHAR. (2018). The IUPHAR/BPS guide to PHARMACOLOGY in 2018: Updates and expansion to encompass the new guide to IMMUNOPHARMACOLOGY. *Nucleic Acids Research*, *46*, D1091–D1106.

Hatanaka, T., Ukai, M., Watanabe, M., Someya, A., Ohtake, A., Suzuki, M., … Kaku, S. (2013). Effect of mirabegron, a novel β_3-adrenoceptor agonist, on bladder function during storage phase in rats. *Naunyn-Schmiedeberg's Archives of Pharmacology*, *386*, 71–78.

Hayashi, M., Kajioka, S., Itsumi, M., Takahashi, R., Shahab, N., Ishigami, T., … Naito, S. (2016). Actions of cAMP on calcium sensitization in human detrusor smooth muscle contraction. *BJU International*, *117*, 179–191.

Hoffmann, C., Leitz, M. R., Oberdorf-Maass, S., Lohse, M. J., & Klotz, K. N. (2004). Comparative pharmacology of human β-adrenergic receptor subtypes—Characterization of stably transfected receptors in CHO cells. *Naunyn-Schmiedeberg's Archives of Pharmacology*, *369*, 151–159. https://doi.org/10.1007/s00210-003-0860-y

Holz, G. G., Kang, G., Harbeck, M., Roe, M. W., & Chepurny, O. G. (2006). Cell physiology of cAMP sensor Epac. *The Journal of Physiology*, *577*, 5–15.

Hucho, T. B., Dina, O. A., & Levine, J. D. (2005). Epac mediates a cAMP-to-PKC signaling in inflammatory pain: an isolectin B4(+) neuron-specific mechanism. *The Journal of Neuroscience*, *25*, 6119–6126.

Hughes, S. J., Cravetchi, X., Vilas, G., & Hammond, J. R. (2015). Adenosine A_1 receptor activation modulates human equilibrative nucleoside transporter 1 (hENT1) activity via PKC-mediated phosphorylation of serine-281. *Cellular Signalling*, *27*, 1008–1018.

Hwang, M., Go, Y., Park, J. H., Shin, S. K., Song, S. E., Oh, B. C., … Song, D. K. (2017). Epac2a-null mice exhibit obesity-prone nature more susceptible to leptin resistance. *International Journal of Obesity*, *41*, 279–288. https://doi.org/10.1038/ijo.2016.208

Hypolite, J. A., Chang, S., Wein, A. J., Chacko, S., & Malykhina, A. P. (2015). Protein kinase C modulates frequency of micturition and non-voiding contractions in the urinary bladder via neuronal and myogenic mechanisms. *BMC Urology*, *15*(34), 1–18. https://doi.org/10.1186/s12894-015-0030-9

Igawa, Y., & Aizawa, N. (2017). Incontinence: How do β_3-adrenoceptor agonists work in the bladder? *Nature Reviews. Urology*, *14*, 330–332.

Igawa, Y., Aizawa, N., & Michel, M. C. (2019). β_3-Adrenoceptors in the normal and diseased urinary bladder—What are the open questions? *Br J Pharmacol*, *176*, 2525–2538. https://doi.org/10.1111/bph.14658

Igawa, Y., Yamazaki, Y., Takeda, H., Hayakawa, K., Akahane, M., Ajisawa, Y., … Andersson, K.-E. (1999). Functional and molecular biological evidence for a possible β3-adrenoceptor in the human detrusor muscle. *British Journal of Pharmacology*, *126*, 819–825.

Kanzler, S. A., Januario, A. C., & Paschoalini, M. A. (2011). Involvement of β_3-adrenergic receptors in the control of food intake in rats. *Brazilian Journal of Medical and Biological Research*, *44*, 1141–1147. https://doi.org/10.1590/s0100-879x2011007500127

Kawasaki, H., Springett, G. M., Mochizuki, N., Toki, S., Nakaya, M., Matsuda, M., … Graybiel, A. M. (1998). A family of cAMP-binding proteins that directly activate Rap1. *Science*, *282*, 2275–2279.

Kilkenny, C., Browne, W., Cuthill, I. C., Emerson, M., & Altman, D. G. (2010). Animal research: Reporting in vivo experiments: The ARRIVE guidelines. *British Journal of Pharmacology*, *160*, 1577–1579.

Kullmann, F. A., Limberg, B. J., Artim, D. E., Shah, M., Downs, T. R., Contract, D., … de Groat, W. C. (2009). Effects of β3-adrenergic receptor activation on rat urinary bladder hyperactivity induced by ovariectomy. *The Journal of Pharmacology and Experimental Therapeutics*, *330*, 704–717.

Leitner, M. G., Michel, N., Behrendt, M., Dierich, M., Dembla, S., Wilke, B. U., … Oliver, D. (2016). Direct modulation of TRPM4 and TRPM3 channels by the phospholipase C inhibitor U73122. *British Journal of Pharmacology*, *173*, 2555–2569.

Li, J., O'Connor, K. L., Cheng, X., Mei, F. C., Uchida, T., Townsend, C. M. Jr., et al. (2007). Cyclic adenosine 5′-monophosphate-stimulated neurotensin secretion is mediated through Rap1 downstream of both Epac and protein kinase A signaling pathways. *Molecular Endocrinology*, *21*, 159–171.

Li, L., Cai, H., Liu, H., & Guo, T. (2015). β-adrenergic stimulation activates protein kinase Cε and induces extracellular signal-regulated kinase phosphorylation and cardiomyocyte hypertrophy. *Molecular Medicine Reports*, *11*, 4373–4380. https://doi.org/10.3892/mmr.2015.3316

Limberg, B. J., Andersson, K. E., Kullmann, A. F., Burmer, G., de Groat, W. C., & Rosenbaum, J. S. (2010). β-Adrenergic receptor

subtype expression in myocyte and non-myocyte cells in human female bladder. *Cell and Tissue Research*, *342*, 295–306.

Lipp, P., & Reither, G. (2011). Protein kinase C: The "masters" of calcium and lipid. *Cold Spring Harbor Perspectives in Biology*, *3*, 1–17, a004556. https://doi.org/10.1101/cshperspect.a004556

Maki, T., Kajioka, S., Itsumi, M., Kareman, E., Lee, K., Shiota, M., & Eto, M. (2019). Mirabegron induces relaxant effects via cAMP signaling-dependent and -independent pathways in detrusor smooth muscle. *Low Urin Tract Symptoms*, *2019*, 1–9. https://doi.org/10.1111/luts.12247

Marcelissen, T., Rashid, T., Antunes-Lopes, T., Delongchamps, N. B., Geavlete, B., Rieken, M., … EAU Young Academic Urologists (YAU) functional urology working group (2019). Oral pharmacologic management of overactive bladder syndrome: Where do we stand? *European Urology Focus*, *5*, 1112–1119. https://doi.org/10.1016/j.euf.2018.03.011

Mayati, A., Moreau, A., Le Vée, M., Stieger, B., Denizot, C., Parmentier, Y., & Fardel, O. (2017). Protein kinases C-mediated regulations of drug transporter activity, localization and expression. *International Journal of Molecular Sciences*, *18*, 1–22, 764. https://doi.org/10.3390/ijms18040764

Michel, M. C., & Korstanje, C. (2016). β3-Adrenoceptor agonists for overactive bladder syndrome: Role of translational pharmacology in a repositioning clinical drug development project. *Pharmacology & Therapeutics*, *159*, 66–82. https://doi.org/10.1016/j.pharmthera.2016.01.007

Michel, M. C., & Vrydag, W. (2006). α1-, α2- and β-adrenoceptors in the urinary bladder, urethra and prostate. *British Journal of Pharmacology*, *147*(Suppl 2), S88–S119.

Moreira-Rodrigues, M., Graça, A. L., Ferreira, M., Afonso, J., Serrão, P., Morato, M., … Moura, D. (2014). Attenuated aortic vasodilation and sympathetic prejunctional facilitation in epinephrine-deficient mice: Selective impairment of β_2-adrenoceptor responses. *The Journal of Pharmacology and Experimental Therapeutics*, *351*, 243–249.

Nakagomi, H., Kullmann, F. A., Ruiz, W. G., Wolf-Johnston, A., Apodaca, G., & Birder, L. A. (2015). Urothelial Epac: Novel mediator in the regulation of bladder capacity. *The Journal of Urology*, *193*(Suppl 4), e233, MP21-04–e234. https://doi.org/10.1016/j.juro.2015.02.956

Niclauss, N., Michel-Reher, M. B., Alewijnse, A. E., & Michel, M. C. (2006). Comparison of three radioligands for the labelling of human β-adrenoceptor subtypes. *Naunyn-Schmiedeberg's Archives of Pharmacology*, *374*, 99–105. https://doi.org/10.1007/s00210-006-0104-z

Obata, T., Kubota, S., & Yamanaka, Y. (2001). Histamine increases interstitial adenosine concentration via activation of ecto-5′-nucleotidase in rat hearts in vivo. *The Journal of Pharmacology and Experimental Therapeutics*, *298*, 71–76.

Oestreich, E. A., Wang, H., Malik, S., Kaproth-Joslin, K. A., Blaxall, B. C., Kelley, G. G., … Smrcka, A. V. (2007). Epac-mediated activation of phospholipase C (epsilon) plays a critical role in β-adrenergic receptor-dependent enhancement of Ca^{2+} mobilization in cardiac myocytes. *The Journal of Biological Chemistry*, *282*, 5488–5495.

Okeke, K., Gravas, S., & Michel, M. C. (2017). Do β_3-adrenoceptor agonists cause urinary bladder smooth muscle relaxation by inhibiting acetylcholine release? *American Journal of Physiology. Renal Physiology*, *313*, F859–F861. https://doi.org/10.1152/ajprenal.00215.2017

Oliveira, L., & Correia-de-Sá, P. (2005). Protein kinase A and Ca(v)1 (L-Type) channels are common targets to facilitatory adenosine A_{2A} and muscarinic M_1 receptors on rat motoneurons. *Neurosignals*, *14*, 262–272.

Otsuka, A., Kawasaki, H., Matsumoto, R., Shinbo, H., Kurita, Y., Iwashita, T., & Ozono, S. (2013). Expression of β-adrenoceptor subtypes in urothelium, interstitial cells and detrusor of the human urinary bladder. *Low Urin Tract Symptoms*, *5*, 173–180. https://doi.org/10.1111/luts.12007

Otsuka, A., Shinbo, H., Matsumoto, R., Kurita, Y., & Ozono, S. (2008). Expression and functional role of β-adrenoceptors in the human urinary bladder urothelium. *Naunyn-Schmiedeberg's Archives of Pharmacology*, *377*, 473–481.

Parkinson, F. E., Xiong, W., & Zamzow, C. R. (2005). Astrocytes and neurons: Different roles in regulating adenosine levels. *Neurological Research*, *27*, 153–160.

Reyes, G., Nivillac, N. M. I., Karim, M. Z., Desouza, L., Siu, K. W. M., & Coe, I. R. (2011). The equilibrative nucleoside transporter (ENT1) can be phosphorylated at multiple sites by PKC and PKA. *Molecular Membrane Biology*, *28*, 412–426.

Schena, G., & Caplan, M. J. (2019). Everything you always wanted to know about β_3-AR (but were afraid to ask). *Cells*, *8*, 1–24, 357. https://doi.org/10.3390/cells8040357

Silva, I., Costa, A. F., Moreira, S., Ferreirinha, F., Magalhães-Cardoso, M. T., Calejo, I., … Correia-de-Sá, P. (2017). Inhibition of cholinergic neurotransmission by β_3-adrenoceptors depends on adenosine release and A_1 receptors activation in human and rat urinary bladders. *American Journal of Physiology. Renal Physiology*, *313*, F388–F403.

Silva-Ramos, M., Silva, I., Faria, M., Magalhães-Cardoso, M. T., Correia, J., Ferreirinha, F., & Correia-de-Sá, P. (2015). Impairment of ATP hydrolysis decreases adenosine A_1 receptor tonus favoring cholinergic nerves in the obstructed human urinary bladder. *Purinergic Signal*, *11*, 595–606.

Sinclair, C. J. D., Shepel, P. N., Geiger, J. D., & Parkinson, F. E. (2000). Stimulation of nucleoside efflux and inhibition of adenosine kinase by A_1 adenosine receptor activation. *Biochemical Pharmacology*, *59*, 477–483.

Takasu, T., Ukai, M., Sato, S., Matsui, T., Nagase, I., Maruyama, T., … Yamaguchi, O. (2007). 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. *The Journal of Pharmacology and Experimental Therapeutics*, *321*, 642–647. https://doi.org/10.1124/jpet.106.115840

Takeda, M., Obara, K., Mizusawa, T., Tomita, Y., Arai, K., Tsutsui, T., … Nomura, S. (1999). Evidence for β_3-adrenoceptor subtypes in relaxation of the human urinary bladder detrusor: Analysis by molecular biological and pharmacological methods. *The Journal of Pharmacology and Experimental Therapeutics*, *288*, 1367–1373.

Tyagi, P., Thomas, C. A., Yoshimura, N., & Chancellor, M. B. (2009). Investigations into the presence of functional β_1, β_2 and β_3-adrenoceptors in urothelium and detrusor of human bladder. *International Braz J Urol*, *35*, 76–83.

Uchida, H., Shishido, K., Nomiya, M., & Yamaguchi, O. (2005). Involvement of cyclic AMP-dependent and -independent mechanisms in the relaxation of rat detrusor muscle via β-adrenoceptors. *European Journal of Pharmacology*, *518*, 195–202.

Vieira, C., Duarte-Araújo, M., Adães, S., Magalhães-Cardoso, T., & Correia-de-Sá, P. (2009). Muscarinic M_3 facilitation of acetylcholine release from rat myenteric neurons depends on adenosine outflow leading to activation of excitatory A_{2A} receptors. *Neurogastroenterology and Motility*, *21*, 1118–e95.

Vliem, M. J., Ponsioen, B., Schwede, F., Pannekoek, W. J., Riedl, J., Kooistra, M. R., … Rehmann, H. (2008). 8-pCPT-2'-O-Me-cAMP-AM: An improved Epac-selective cAMP analogue. *Chembiochem*, *9*, 2052–2054. https://doi.org/10.1002/cbic.200800216

Wang, P., Liu, Z., Chen, H., Ye, N., Cheng, X., & Zhou, J. (2017). Exchange proteins directly activated by cAMP (EPACs): Emerging therapeutic targets. *Biorg Med Chem Lett*, *27*, 1633–1639.

Winder, M., Tobin, G., Zupančič, D., & Romih, R. (2014). Signaling molecules in the urothelium. *Biomed Res Int*, *2014*, 1–14. https://doi.org/10.1155/2014/297295

Woods, M., Carson, N., Nortan, N. W., Sheldon, J. H., & Argentieri, T. M. (2001). Efficacy of the β3-adrenergic receptor agonist CL316243 on

experimental bladder hyperreflexia and detrusor instability in the rat. *The Journal of Urology*, *166*, 1142–1147.

Wuest, M., Eichhorn, B., Grimm, M. O., Wirth, M. P., Ravens, U., & Kaumann, A. J. (2009). Catecholamines relax detrusor through β 2-adrenoceptors in mouse and β 3-adrenoceptors in man. *The Journal of Pharmacology and Experimental Therapeutics*, *328*, 213–222. https://doi.org/10.1124/jpet.108.142562

Yamaguchi, O., & Chapple, C. R. (2007). β3-adrenoceptors in urinary bladder. *Neurourology and Urodynamics*, *26*, 752–756.

Yamazaki, Y., Takeda, H., Akahane, M., Igawa, Y., Nishizawa, O., & Ajisawa, Y. (1998). Species differences in the distribution of β-adrenoceptor subtypes in bladder smooth muscle. *British Journal of Pharmacology*, *124*, 593–599.

Yoshida, M., Miyamae, K., Iwashita, H., Otani, M., & Inadome, A. (2004). Management of detrusor dysfunction in the elderly: Changes in acetylcholine and adenosine triphosphate release during aging. *Urology*, *63*(3 Suppl 1), 17–23.

Zhu, Y., Chen, H., Boulton, S., Mei, F., Ye, N., Melacini, G., … Cheng, X. (2015). Biochemical and pharmacological characterizations of ESI-09 based EPAC inhibitors: Defining the ESI-09 "Therapeutic Window". *Scientific Reports*, *5*, 1–8, 9344. https://doi.org/10.1038/srep09344

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