Cellular Physiology and Biochemistry

Cell Physiol Biochem 2018;48:847-862
DOI: 10.1159/000491916
Published online: July 20, 2018
Accepted: May 20, 2018

© 2018 The Author(s)
Published by S. Karger AG, Basel
www.karger.com/cpb

Karger Open access

This article is licensed under the Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License (CC BY-NC-ND) (http://www.karger.com/Services/OpenAccessLicense). Usage and distribution for commercial purposes as well as any distribution of modified material requires written permission.

Original Paper

Human β_3-Adrenoreceptor is Resistant to Agonist-Induced Desensitization in Renal Epithelial Cells

Serena Milano[a] Andrea Gerbino[a] Giorgia Schena[b] Monica Carmosino[b] Maria Svelto[a] Giuseppe Procino[a]

[a]Department of Biosciences, Biotechnologies and Biopharmaceutics, University of Bari, Bari, [b]Department of Sciences, University of Basilicata, Potenza, Italy

Key Words
β_3-adrenoreceptor • Mirabegron • cAMP and Ca^{2+} signaling • Desensitization • Internalization • Downregulation • Type-2 vasopressin receptor • Aquaporin 2 • Nephrogenic diabetes insipidus

Abstract
Background/Aims: We recently showed that the β_3-adrenoreceptor (β_3AR) is expressed in mouse kidney collecting ducts (CD) cells along with the type-2 vasopressin receptor (AVPR2). Interestingly, a single injection of a β_3AR selective agonist promotes a potent antidiuretic effect in mice. Before considering the feasibility of chronic β_3AR agonism to induce antidiuresis *in vivo*, we aimed to evaluate *in vitro* the signaling and desensitization profiles of human β_3AR. ***Methods:*** Human β_3AR desensitization was compared with that of human AVPR2 in cultured renal cells. Video imaging and FRET experiments were performed to dissect β_3AR signaling under acute and chronic stimulation. Plasma membrane localization of β_3AR, AVPR2 and AQP2 after agonist stimulation was studied by confocal microscopy. Receptors degradation was evaluated by Western blotting. ***Results:*** In renal cells acute stimulation with the selective β_3AR agonist mirabegron, induced a dose-dependent increase in cAMP. Interestingly, chronic exposure to mirabegron promoted a significant increase of intracellular cAMP up to 12 hours. In addition, a slow and slight agonist-induced internalization and a delayed downregulation of β_3AR was observed under chronic stimulation. Furthermore, chronic exposure to mirabegron promoted apical expression of AQP2 also up to 12 hours. Conversely, long-term stimulation of AVPR2 with dDAVP showed short-lasting receptor signaling, rapid internalization and downregulation and apical AQP2 expression for no longer than 3 h. ***Conclusions:*** Overall, we conclude that β_3AR is less prone than AVPR2 to agonist-induced desensitization in renal collecting duct epithelial cells, showing sustained cAMP production, preserved membrane localization and delayed degradation after 12 hours agonist exposure. These results may be important for the potential use of chronic pharmacological stimulation of β_3AR to promote antidiuresis overcoming *in vivo* renal concentrating defects caused by inactivating mutations of the AVPR2.

© 2018 The Author(s)
Published by S. Karger AG, Basel

S. Milano and A. Gerbino contributed equally to this work.

Prof. Giuseppe Procino,
Assistant Professor of
Physiology

Department of Biosciences, Biotechnologies and Biopharmaceutics, University of Bari
Bari (Italy)
Tel. +39-080-5443414, E-Mail giuseppe.procino@uniba.it

Introduction

β_3-adrenoreceptor (β_3AR) is a G-protein coupled receptor (GPCR) discovered and cloned in the 1980s [1-3]. Early studies demonstrated that human β_3AR is expressed primarily in both white and brown adipocytes where regulates lipolysis and thermogenesis [2]. More recent reports indicate that β_3AR is also expressed in human heart, gallbladder, gastrointestinal tract, prostate and urinary bladder detrusor, brain as well as in near-term myometrium [4].

We recently showed, for the first time that, the β_3AR is also expressed in different nephron segments of mouse kidney. In the cortical and outer medullary collecting duct, β_3AR shares its localization with the vasopressin type-2 receptor (AVPR2), whose activation by vasopressin (AVP) is critical for the shuttling of the water channel aquaporin 2 (AQP2) at the apical plasma membrane, increase of water reabsorption and, ultimately, antidiuresis. We proved, in fact, that short-term β_3AR selective agonism promoted a potent antidiuretic effect in mice lacking AVPR2 [5].

These results are relevant when considering β_3AR as a potential drug target for the treatment of X-linked Nephrogenic Diabetes Insipidus (X-NDI) [6-9], a congenital disease caused by inactivating mutations of the AVPR2 and consequential lack of AVP-induced antidiuresis.

To consider a possible chronic use of β_3AR agonists as therapeutic approach for X-NDI it is mandatory to evaluate whether and when the receptor undergoes desensitization switching off its downstream signaling with reduction of agonist response and clinical efficacy of the drug. In general, desensitization of a GPCR results in decreased responsiveness to repeated or chronic exposure to agonist. Desensitization mechanisms include: 1) receptor uncoupling from downstream signal transduction elements mediated by kinases; 2) receptor internalization; 3) receptor down-regulation via increased degradation and/or reduced synthesis [10-12].

It is well established that agonist-promoted desensitization of both β_1- and β_2-adrenoreceptors is mainly due to phosphorylation of the receptors by several kinases including the β adrenoreceptor kinase (βARK) and the cAMP-dependent protein kinase A (PKA) [13-15]. β_3AR, unlike β_1- and β_2-AR, lacks the target amino acid sequences for βARK and PKA in the cytoplasmic C-terminus.

Consequently, β_3AR should be less susceptible to PKA/βARK-mediated receptor recycling and desensitization in response to hyperstimulation [16-18].

Still, the question whether the β_3AR is subject to desensitization is a matter of debate. On one hand, it has been shown that β_3AR transfected into Chinese hamster ovary (CHO) cells [14, 19, 20], murine L fibroblast cells and chinese hamster fibroblast (CHW) cells [18, 21] or endogenously expressed in human SK-N-MC neuroblastoma cells [16] was resistant to both short- and long-term agonist-induced desensitization. Experiments to investigate desensitization in rodent adipose tissue did not shed a light on this controversial picture [13, 22-25]. On the other hand, agonist-induced β_3AR desensitization was demonstrated in mouse ileum [24] and with human embryonic kidney (HEK) cells transfected with β_3AR [14, 26].

Overall, these findings suggest that agonist-induced desensitization of β_3-adrenoceptors occurs in a tissue- or cell type-dependent manner [27].

Therefore, the present study was designed to specifically explore human β_3AR desensitization dynamics in renal collecting duct epithelial cells, which have not been described so far. The evaluation of long-term desensitization could be important for unveiling the potential tachyphylaxis occurring during chronic treatment of X-NDI with β_3AR agonists. In this respect, we analyzed signal transduction pathways, membrane trafficking and expression level of β_3AR chronically stimulated with the specific agonist mirabegron which has been already introduced into the clinic for treatment of overactive bladder syndrome [28, 29].

To this end, we stably expressed the β_3AR tagged with mCherry in mouse collecting duct M-1 cells [30] and compared these results with data from M-1 cells expressing human AVPR2, whose desensitization dynamics were well characterized in the collecting duct [31-34].

Here, we found that β_3AR is less prone than AVPR2 to agonist-induced desensitization in renal collecting duct epithelial cells, showing sustained cAMP production, persistent membrane localization and delayed degradation under long-term agonist exposure. Of note, the sustained cAMP signaling induced by β_3AR activation deeply impact AQP2 membrane localization, which is continuously expressed on the apical membrane for up to 12 h.

These results encourage preclinical studies supporting the hypothesis that chronic mirabegron treatment could promote a durable antidiuretic effect in X-NDI patients.

Materials and Methods

Antibodies and Reagents

1-deamino-8-D-arginine vasopressin (dDAVP, cat.# V-1005) and cycloheximide (cat.# c1988) were from Sigma (www.sigmaaldrich.com), while mirabegron (cat.# sc211912) was from Santa Cruz Biotechnology (www.scbt.com). Mouse monoclonal anti-Na^+/K^+-ATPase α-1 antibody (cat.# 05-369) was from Millipore (www.merckmillipore.com), rabbit anti-mCherry (cat.# ab167453) from Abcam (www.abcam.com) and polyclonal anti-FLAG® (cat.# F7425) from Sigma. The rabbit affinity-purified polyclonal antibody against human AQP2 was previously described [35].

cDNA constructs of β_3AR-mCh/ FLAG® *and AVPR2-mCh*

Human β_3-adrenoreceptor (β_3AR), cloned in pcDNA3.1 plasmid, and human vasopressin type 2 receptor (AVPR2), cloned in pRP vector, were from ThermoFisher (www.thermofisher.com) and Cyagen (www.cyagen.com), respectively. The fluorescent tag mCherry was attached to the carboxy-terminus of both receptors. The human β_3AR cloned in pRP vector tagged with FLAG® at C-terminus was from Cyagen.

Cell culture and transfection

Mouse cortical collecting duct M-1 cells [30] and MCD4 cells, a clone of M-1 cells stably transfected with human- AQP2, were cultured as described elsewhere [36, 37]. To obtain stable cell lines expressing β_3AR-mCh (M-1-β_3AR-mCh and MCD4-β_3AR-mCh) or AVPR2-mCh (M-1-AVPR2-mCh and MCD4-AVPR2-mCh) cells were transfected using Lipofectamine® 2000 (www.thermofisher.com) and the appropriate construct according to the manufacturer's instructions. After 14-20 days of selection in medium containing 500 μg/ml hygromicin B (www.thermofisher.com), resistant colonies were isolated with cloning rings and expanded to evaluate expression and membrane localization of β_3AR-mCh or AVPR2-mCh. In parallel experiments M-1 cells were transiently transfected using Lipofectamine® 2000 with β_3AR-FLAG®.

FRET-based measurement of cAMP/PKA in single cells

The FRET-based cAMP sensor Epac H96 [38] was a generous gift of Prof. Kees Jalink (Netherlands Cancer Institute, Amsterdam, Netherlands). AKAR4 [39] was kindly provided by Prof. Jin Zhang (John Hopkins University School of Medicine, Baltimore, MD). Cells were seeded on Ø 25 mm glass coverslips and transiently transfected with Epac H96 or AKAR4 using Lipofectamine® 2000 (www.thermofisher.com). Cells were mounted in a perfusion chamber (FCS2 Closed Chamber System, www.bioptechs.com) and imaged using 40× Plan Fluor (NA 1.30) oil immersion objective lens. An Hepes-buffered Ringer's solution was used to perfuse cells during the experiment, containing: 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM Hepes, 5 mM Glucose, 1.0 mM $CaCl_2$, pH 7.4.

Real-time FRET imaging experiments were performed at room temperature using a fluorescence ratio imaging system built around a TE2000-S inverted fluorescence microscope equipped with a cooled CCD camera (CoolSNAP HQ, www.photometrics.com). MetaFluor software (www.moleculardevices.com) was used to acquire ratio data. FRET emission ratios (535 nm/485 nm, 440 nm excitation) were acquired every 5 s. The fluorescence of mCherry (excitation 585 nm, emission 610 nm) did not interfere with any of these measurements [40]. Figures depict data from 5–8 cells summarized for each experiment; at least four independent runs were conducted. Data are expressed as means ± SEM.

Steady state FRET experiments were performed to measure intracellular cAMP levels in single cells using Epac H96 [38], as previously described [35, 41].

Briefly, M-1-β_3AR-mCh and M-1-AVPR2-mCh cells were grown onto Ø 25 mm glass coverslips at 37°C and transfected with Epac H96 using Lipofectamine® 2000. 12 hours before the experiment cells were treated overnight with 50 μM indomethacin, a cyclooxygenase inhibitor, to prevent the increase in basal cAMP concentration due to autocrine/paracrine stimulation of P2-purinergic receptors [42, 43], as previously reported [36]. M-1-β_3AR-mCh and M-1-AVPR2-mCh cells were either left under basal condition or stimulated for 1 h at 37°C with increasing doses of mirabegron or dDAVP: 1 nM, 10 nM, 100 nM and 1 μM.

To evaluate the effect of long-term stimulation of β_3AR-mCh/FLAG® or AVPR2-mCh on cAMP production, Epac H96-expressing cells were pretreated for 30 min with 10 μg/ml cycloheximide, a protein synthesis inhibitor, to prevent the potential contribution of newly synthesized receptor. Cells were either left under basal condition or treated with 10 nM mirabegron or 100 nM dDAVP for 1, 3 ,6, 12 and 24 h at 37°C in presence of 10 μg/ml cycloheximide. After stimulation, cells were fixed in PBS containing 4% paraformaldehyde and mounted on glass slides.

Steady state FRET measurements were carried out using MetaMorph software (www.moleculardevices.com). Data from 15 different fields, each one containing at least 3 H96-expressing cells, were summarized for a single coverslip/treatment, and at least three independent coverslips were blind-analyzed. Averaged netFRET values from the different experimental conditions were normalized as percentage of the netFRET of unstimulated cells. netFRET data were analyzed using Prism software (version 5.0; www.graphpad.com). Unpaired data were assessed for statistical significance using the Student's t test. Data are expressed as means ±SEM and $P < 0.05$ was considered statistically significant.

Evaluation of cytosolic levels with Fura-2

For intracellular Ca^{2+} measurements, M-1-β_3AR-mCh or M-1-AVPR2-mCh cells were seeded on glass coverslips (Ø 25 mm). Ringer's Solution was used to perfuse cells during the experiment containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM Hepes, 5 mM Glucose, 1.0 mM $CaCl_2$, pH 7.4. Cells were loaded with 5 μM Fura-2 (www.thermofisher.com) for 25 min at 37°C in DMEM. Coverslips with dye-loaded cells were analyzed with a set-up described in detail in previous papers of our group [41, 44, 45]. Fluorescence measurements were performed using Metafluor (www.moleculardevices.com). After background images correction, Fura-2 ratio was normalized to an average 2 min. baseline (R0) before agonist addition to obtain the relative R/R_0. Figure depict 4 different cells of the same coverslip, at least four independent runs were conducted.

Immunofluorescence

As already described for steady-state FRET experiments, M-1-β_3AR-mCh/FLAG®, MCD4-β_3AR-mCh, M-1-AVPR2-mCh or MCD4-AVPR2-mCh cells were treated with 10 μg/ml cycloheximide and stimulated for 1, 3 ,6 and 12 h with 10 nM mirabegron or 100 nM dDAVP, respectively. Cells treated with 10 μg/ml cycloheximide alone for 30 min were considered as resting condition. Cell monolayers were then fixed in PBS containing 4% paraformaldehyde (M-1-β_3AR-mCh/ FLAG® and M-1-AVPR2-mCh cells) or methanol (MCD4-β_3AR-mCh and MCD4-AVPR2-mCh cells) and washed twice with PBS. Cells were blocked with 1% BSA in PBS, then anti-Na^+/K^+-ATPase α-1 antibody and anti-FLAG® (for M-1-β_3AR-mCh/FLAG® and M-1-AVPR2-mCh) or anti-AQP2 antibody (for MCD4-β_3AR-mCh and MCD4-AVPR2-mCh cells) were incubated for 2 h at room temperature. The bound antibodies was detected with Alexa Fluor secondary antibodies (www.thermofisher.com). Images were acquired with a Leica TCS-SP2 confocal microscope (www.leica-microsystems.com).

Western blotting

These experiments were performed in presence of 10 μg/ml cycloheximide, as reported above. M-1-β_3AR-mCh and M-1-AVPR2-mCh cells grown in 6-well culture plates were either left under basal condition or treated for 1, 3 ,6, 12 and 24 h at 37°C with 10 nM mirabegron or 100 nM dDAVP, respectively. Cells were washed twice with ice-cold PBS w/o Ca^{2+} and Mg^{2+} and scraped into RIPA buffer (150 mM NaCl, 10 mM Tris, pH 7.2, 0.1% SDS, 1% Triton X-100, 1% deoxycholate, 5 mM EDTA) containing protease and phosphatase inhibitors (1 mmol/l phenylmethylsulfonyl fluoride, 10 mmol/l leupeptin, 1 mg/ml pepstatin A, 10 mmol/l NaF, 1 mmol/l sodium orthovanadate, and 15 mmol/l tetrasodium pyrophosphate). Lysates were then

sonicated for 30 seconds at 4°C using a microprobe sonicator. Insoluble material was pelleted at 13, 000 g for 30 min at 4°C. 5 µg of each supernatant was separated by standard SDS-PAGE using Mini-PROTEAN® TGX Stain-Free™ Precast Gels Bio-Rad and analyzed by Western blotting as previously described [46]. Densitometry was performed using the Image Lab™ software (Bio-rad) bundled with ChemiDoc™(Bio-Rad) imaging system, after normalization for the total protein loading using the Stain-Free™ technology (Bio-Rad) according to manufacturer's instructions.

Results

β_3AR activation elevates intracellular cAMP levels, activates PKA and increases Ca^{2+} in renal epithelial cells

At first, we studied the β_3AR signaling in M-1 cells stably expressing the receptor tagged with mCherry (M-1-β_3AR-mCh, Fig. 1A). β_3AR is mainly localized on the plasma membrane, thus putatively able to sense and respond to extracellular stimuli. Thus, to asses β_3AR intracellular signaling, we evaluated cAMP generation and PKA activity in M-1-β_3AR-mCh cells by expressing, either the cytosolic cAMP sensor Epac H96 [38] or the PKA activity probe AKAR4 [39], respectively. Both sensors are FRET-based, however, while Epac H96 works on the principle of "loss of FRET" and its FRET ratio decreases in response to cAMP elevation, AKAR4 works based on "gain of FRET" and reaches maximum FRET ratio when PKA activity is high [47]. Fig. 1 shows that treatment with 10 nM mirabegron, a selective human β_3AR agonist, induced a decrease in the FRET ratio of H96 (Fig. 1B) and a parallel increase in the FRET ratio of AKAR4 (Fig. 1C), indicating increases of both cAMP level and PKA activity only in M-1-β_3AR-mCh cells (white triangles) compared to untransfected cells (black diamonds).

In addition, we investigated whether mirabegron triggers Ca^{2+} oscillations. Fig. 1D shows the simultaneous time courses of the normalized Fura-2 Ratio in four M-1-β_3AR-mCh cells of the same coverslip. Stimulation with 10 nM mirabegron induced not synchronous Ca^{2+} transients with different amplitudes and oscillatory frequencies.

Dose-dependent response to Mirabegron of M-1-β_3AR-mCh cells

Epac H96 was also used in steady-state FRET experiments to determine the lowest dose of mirabegron able to induce a significant cAMP production in M-1-β_3AR-mCh cells (Fig. 2A).

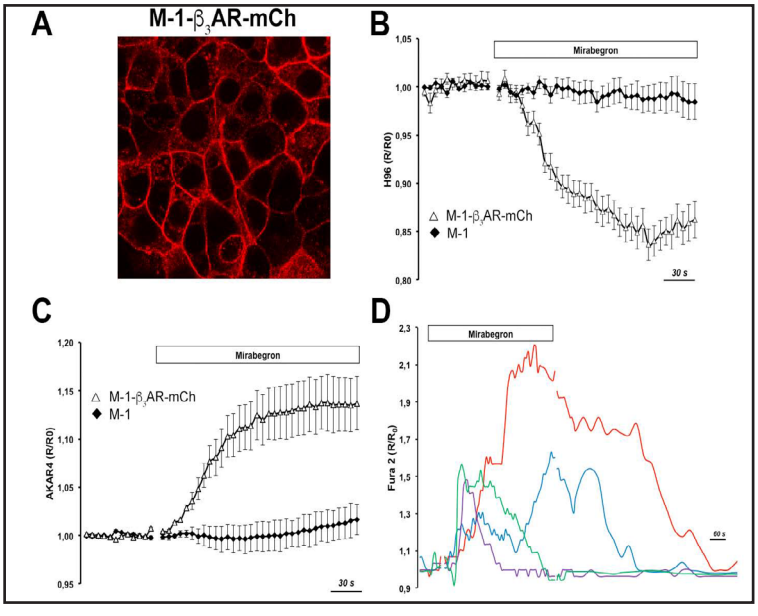

Fig. 1. β_3AR intracellular signaling measured using FRET-based reporters and Fura-2. A) Confocal images of M-1 cells stably expressing β_3AR-mCh. B) M-1 cells either expressing β_3AR-mCh (withe triangles) or not (black diamonds) were transfected with Epac H96 and stimulated with mirabegron 10 nM. Traces represent data from 5–8 cells of the same coverslip. Data are expressed as means ± SEM. C) M-1 cells either expressing β_3AR (withe triangles) or not (black diamonds) were transfected with AKAR4 and stimulated with mirabegron 10 nM. Traces represent data from 5–8 cells of the same coverslip. Data are expressed as means ± SEM. D) Normalized time course of Fura-2 excitation ratio from four different M-1-β_3AR-mCh cells after exposure to 10 nM mirabegron.

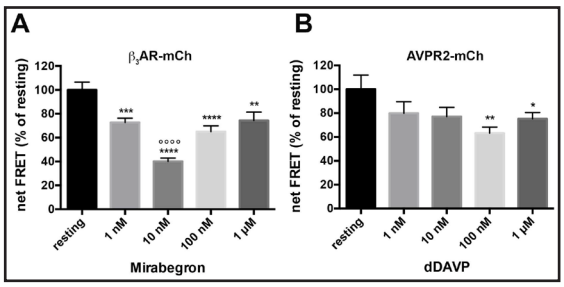

Fig. 2. Dose-response curves of mirabegron in M-1-β_3AR-mCh and dDAVP in M-1-AVPR2-mCh cells determined by Steady-state FRET. A) M-1-β_3AR-mCh and B) M-1-AVPR2-mCh cells were transfected with Epac H96 and stimulated for 1 h at 37°C with 1 nM, 10 nM, 100 nM and 1 μM mirabegron or dDAVP, respectively. cAMP levels inversely correlate with netFRET signals. Data from 15 different fields each one containing at least 3 cells were summarized for a single coverslip/ treatment, and at least three independent coverslips were blind-analyzed. Averaged netFRET values from the different experimental conditions were expressed as percentage of the resting cells. Unpaired data were assessed for statistical significance using the Student's t test. Data are expressed as means ±SEM. *p< 0.05; **p< 0.01; ***p< 0.001; ****p< 0.0001. °°°°p<0.0001 (1 nM vs 10 nM).

One-hour exposure to mirabegron (1 nM, 10 nM, 100 nM or 1 μM) induced a significant cAMP production, when compared to unstimulated cells, for all the tested doses. 10 nM was the lower dose inducing maximal cAMP generation. Accordingly, we used 10 nM mirabegron throughout this study.

Parallel experiments were performed in M-1-AVPR2-mCh cells to evaluate the minimum effective dose of dDAVP, a selective AVPR2 agonist. Fig. 2B shows that intracellular cAMP levels did not significantly change after treatment with 1 nM and 10 nM dDAVP while cAMP production was maximal upon exposure to 100 nM dDAVP, as already reported [32, 48, 49], which was hence the concentration used for all the following experiments.

Sustained receptor stimulation does not turn off the β_3AR signaling

Next, we evaluated if long-term exposure to mirabegron desensitizes β_3AR in term of cAMP signaling in M-1-β_3AR-mCh cells expressing Epac H96. We calculated steady state netFRET changes as index of the cAMP production induced by mirabegron over different time points (1, 3, 6, 12 and 24 hours). All experiments were performed in presence of cycloheximide to inhibit protein synthesis and thus excluding the potential contribution of newly synthesized receptors to cAMP production.

Compared to resting cells (cycloheximide, Fig. 3A), the netFRET signal was significantly reduced (of about a 30%) in M-1-β_3AR-mCh cells treated with mirabegron for 1 h, indicating cAMP generation. This significant decrease of netFRET was also observed in cells exposed to mirabegron up to 12 hours. These findings indicate long-term, continuous cAMP signaling in the presence of mirabegron and suggest that no β_3AR desensitization occurred within 12 h. To check whether this sustained response could be an artifact due to the presence of the mCherry tag, we repeated the same experiment in M-1 cells transiently transfected with FLAG®-β_3AR (M-1-β_3AR-FLAG®, Fig. 3B). The results obtained with the two β_3AR constructs were overlapping.

Parallel experiments performed on M-1-AVPR2-mCh cells showed that 1h exposure to dDAVP elicited a significant reduction in netFRET when compared to untreated cells (cycloheximide, Fig. 3C). This netFRET reduction was of the same extent of that induced by mirabegron in M-1-β_3AR-mCh/ FLAG® cells, indicating similar cAMP signaling after 1h of agonist exposure in both cell lines. However, dDAVP stimulation longer that 1h did not induce any significant netFRET reduction. This evidence likely suggested that β_3AR was less prone than AVPR2 to undergo long-term agonist-induced desensitization in renal collecting duct.

Plasma membrane localization of β_3AR under chronic agonist stimulation in renal epithelial cells

Next, we analyzed the cellular distribution of the receptor in M-1-β_3AR-mCh cells upon chronic stimulation with mirabegron in the presence of cycloheximide to exclude

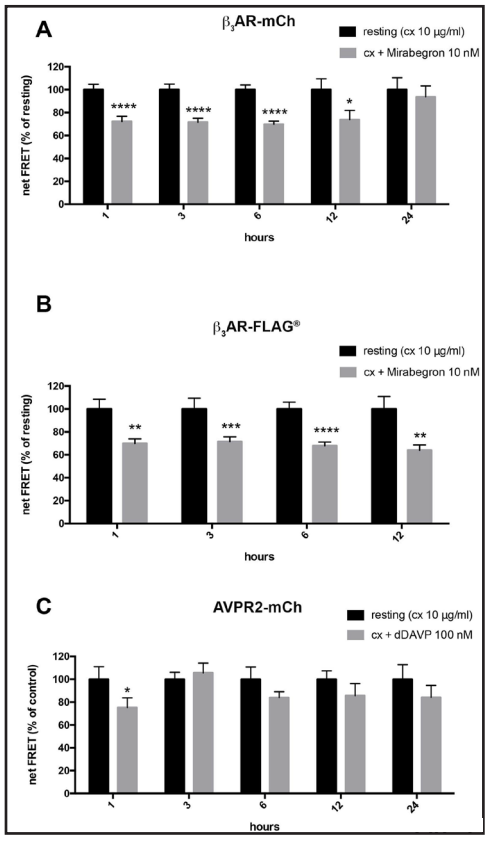

Fig. 3. Long-term exposure to the agonist does not desensitize β_3AR whereas turns off the AVPR2 response. A) M-1-β_3AR-mCh cells were treated for 30 min with cycloheximide and next were either left under basal condition (10 µg/ml cycloheximide) or treated with cycloheximide at 37°C with 10 nM mirabegron for 1, 3 ,6, 12 and 24 h. Steady state FRET experiments performed with Epac H96 demonstrate that long-term exposure to mirabegron promoted a significant increase in intracellular cAMP for up to 12 hours. B) M-1 cells transiently transfected with FLAG®-tagged human β_3AR (M-1-β_3AR-FLAG®) were treated for 30 min with cycloheximide, then were either left under basal condition (10 µg/ml cycloheximide) or treated at 37°C with 10 nM mirabegron for 1, 3 ,6 and 12 h in the presence cycloheximide. Steady state FRET experiments performed with Epac H96 demonstrated that long-term stimulation induced a significant increase in intracellular cAMP for up to 12 hours. C) M-1-AVPR2-mCh cells were treated as in A) with dDAVP and intracellular cAMP was measured. dDAVP long-term stimulation induced short-lasting AVPR2 signaling not longer than 1 h. Averaged netFRET values from the different experimental conditions were expressed as percentage of the resting cells at each time points. Comparable results were obtained in 3 different experiments and significance calculated by the Student's t test for unpaired data. Data are expressed as means ±SEM. *p< 0.05; **p<0.01; ***p<0.001; ****p< 0.0001.

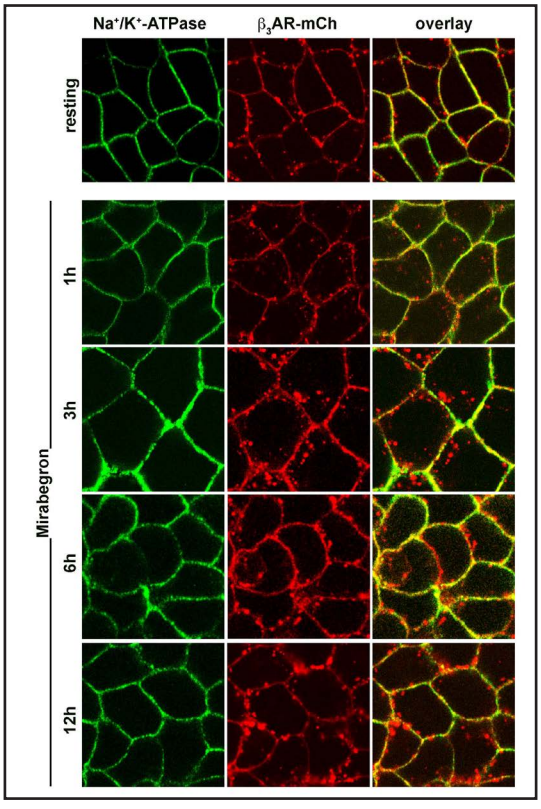

Fig. 4. Cellular localization of β_3AR under chronic agonist stimulation in renal epithelial cells. M-1-β_3AR-mCh cells were treated for 30 min with 10 µg/ml cycloheximide (resting) or stimulated with 10 nM mirabegron for 1, 3, 6 and 12 hours in presence of 10 µg/ml cycloheximide. β_3AR (red) localization was analyzed directly using confocal microscopy. Na^+/K^+-ATPase (green) was used as plasma membrane marker. Mirabegron stimulation produced very little effect on the subcellular distribution of β_3AR. β_3AR predominantly colocalized at the basolateral plasma membrane with Na^+/K^+-ATPase in resting condition and after 1 h of exposure to mirabegron. After 3 and 6 hours of stimulation, β_3AR was predominantly localized at the plasma membrane and appeared in some intracellular vesicles. Remarkably, even 12 h after agonist exposure, β_3AR membrane localization was still partially preserved. Scale bars, 20 µm.

the contribution of newly synthesized receptor (Fig. 4). Confocal microscopy experiments showed that at rest, β_3AR was mainly localized at the basolateral plasma membrane where it largely colocalized with the plasma membrane marker Na^+/K^+-ATPase. After 1 h stimulation with mirabegron, no apparent receptor internalization was observed since β_3AR still largely co-localized with Na^+/K^+-ATPase. At later time points of continuous mirabegron stimulation (3, 6 and 12 h), a modest internalization of the receptor into a cytosolic punctate pattern appeared. However, β_3AR was still predominantly localized at the basolateral plasma membrane, even after 12 h of exposure to mirabegron. To check whether the reduced endocytosis could be explained by the presence of the mCherry tag, we repeated the same experiment with M-1-β_3AR-FLAG® (Fig. 5). The results obtained with the two β_3AR tags were overlapping.

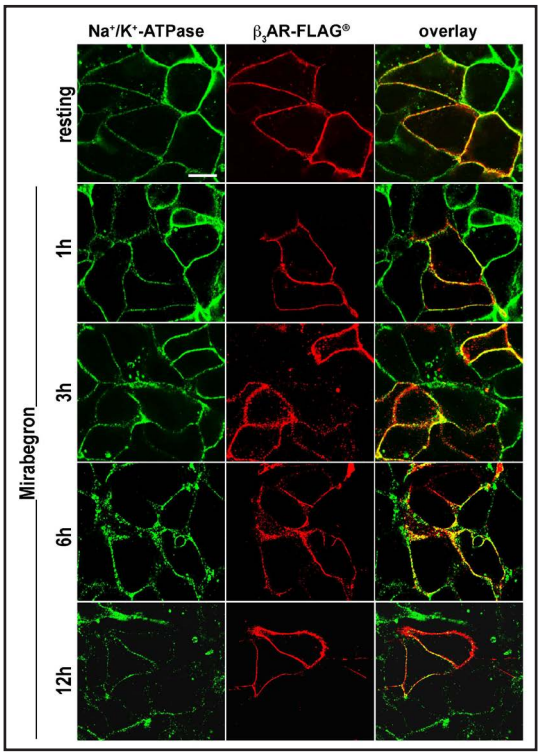

Fig. 5. M-1-β_3AR-FLAG® were treated for 30 min with 10 µg/ml cycloheximide (resting) or stimulated with 10 nM mirabegron for 1, 3, 6 and 12 hours in presence of cycloheximide. Confocal images showed β_3AR-FLAG® in red and Na^+/K^+-ATPase in green. Mirabegron stimulation did not change the subcellular localization of β_3AR-FLAG® even after 12 h of agonist exposure. Images are representative of 3 independent experiments. Scale bars 20 µm.

In parallel experiments, we analyzed the receptor membrane trafficking in M-1-AVPR2-mCh cells. We found that under basal conditions, the AVPR2 was mostly localized at the plasma membrane. In contrast, as shown in Fig. 6, exposure to dDAVP caused a robust and time-dependent internalization of AVPR2, as evidenced by appearance of packed cytoplasmic vesicles (Fig. 6). These effects were already observed at 1 h of incubation with dDAVP. Receptor internalization was more dramatic at later time points and most of the receptors localized within cytoplasmic vesicles rather than at the plasma membrane. In addition, 12 h of continuous exposure to dDAVP led to a strong reduction of AVPR2 signal, probably indicating receptor degradation.

These findings indicated that, unlike AVPR2, β_3AR did not internalize under chronic agonist stimulation in renal epithelial cells.

Long-term receptor stimulation causes delayed downregulation of β_3AR protein in renal collecting duct epithelial cells

Long-term exposure to agonists can cause receptor downregulation, which is characterized by the depletion of the cellular receptor content due to alterations in the rate of receptor degradation and/or synthesis [12]. Here, Western blot experiments were performed in the presence of cycloheximide.

As shown in Fig. 7A anti-mCherry antibodies revealed a band of about 70 kDa for the chimeric β_3AR-mCh. Compared to resting cells (cycloheximide alone), comparable levels of the receptor were observed at 1 and 3 h, while a small reduction of β_3AR-mCh (of about 20%) was observed at 6 and 12 h of continuous receptor activation (Fig. 7B). These results likely indicated that β_3AR-mCh expression was slightly affected by long-term exposure to mirabegron up to 12 hours. Indeed, chronic exposure to mirabegron induced about 70% of receptor downregulation only after 24 h (Fig. 7B).

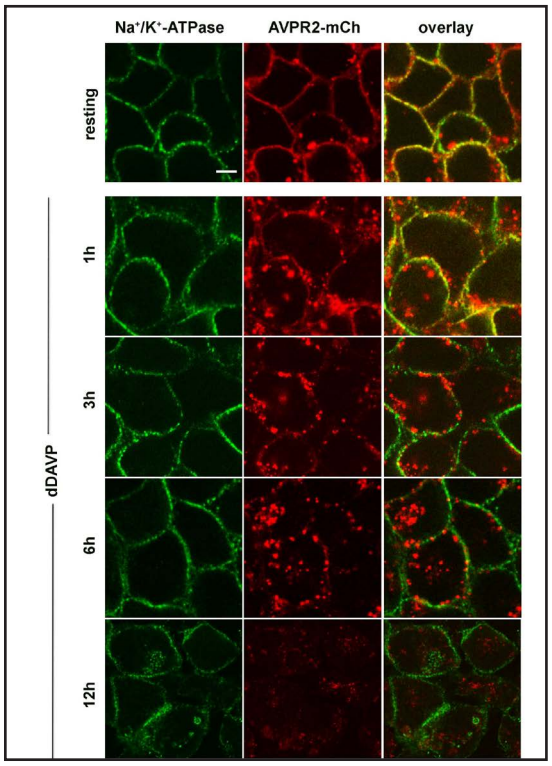

Fig. 6. Cellular localization of AVPR2 under chronic agonist stimulation in renal epithelial cells. M-1-AVPR2-mCh cells were treated for 30 min with 10 µg/ml cycloheximide (resting) or stimulated with 100 nM dDAVP 1, 3, 6 and 12 hours in presence of 10 µg/ml cycloheximide. AVPR2 (red) localization was analyzed directly using confocal microscopy. Na^+/K^+-ATPase (green) was used as plasma membrane marker. Already after 1 h dDAVP, AVPR2 was internalized. At 3 and 6 hours after dDAVP exposure AVPR2 was lost from the plasma membrane and at 12 h receptor membrane staining was strongly reduced, probably because the sustained stimulation of AVPR2 induced receptor degradation. Images are representative of 3 independent experiments. Scale bars, 20 µm.

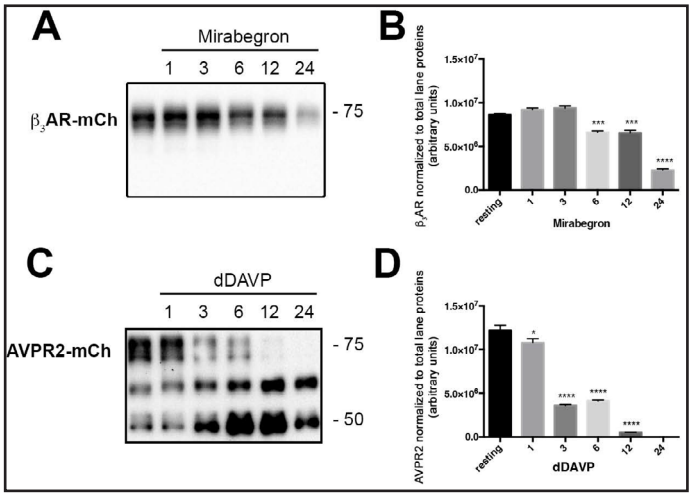

Fig. 7. Effect of long-term agonist stimulation on β_3AR and AVPR2 expression levels. M-1-β_3AR-mCh and M-1-AVPR2-mCh cells were either left under resting condition (10 µg/ml cycloheximide alone; first lane of each blot) or stimulated at 37 °C with 10 nM mirabegron (A) or 100 nM dDAVP (C) for 1, 3, 6, 12 and 24 hours in presence of 10 µg/ml cycloheximide. Cells were lysed and subjected to Western blot analysis to detect, using anti-mCherry antibodies, the expression levels of β_3AR or AVPR2 normalized to total lane protein levels using the Stain-Free™ gels technology. A) Western blotting with anti-mCherry antibodies revealed a band of about 70 kDa for the chimeric β_3AR-mCh. B) Densitometric analysis of β_3AR bands, normalized to total lane proteins, showed no significant differences between resting cells and after 1 and 3 hours of mirabegron treatment. A small reduction of β_3AR (of about 20%) was observed at 6 and 12 h of continuous receptor activation. Mirabegron induced about 70% of receptor downregulation only after 24 h. The experiment was repeated three times and comparable results were obtained. Data are provided as mean ± SEM. ***P<0.001; ****P<0.0001. C) Western blotting with anti-mCherry antibodies revealed a broad smear around 70 kDa band for the mature form of the chimeric AVPR2. Lower bands between 70 and 50 kDa likely represent degradation products of the receptor. D) Densitometric analysis of AVPR2 mature form, normalized to total lane proteins, showed a strong reduction of about 70% even after 3 h exposure to dDAVP and increased at 6 and 12 h. At 24 h of dDAVP stimulation the mature form of AVPR2 was undetectable. On the contrary, the intensity of the lower molecular mass bands, likely representing degradation products of AVPR2, was increased in a time-dependent manner. These results are representative of 3 independent experiments. Data are provided as mean ± SEM. *P<0.05; ****P<0.0001.

Conversely, in M1-AVPR2-mCh cells, the anti-mCherry antibody revealed a broad smear around 70 kDa and lower bands between 70 and 50 kDa (Fig. 7C). In presence of dDAVP, the intensity of higher molecular mass bands, representing the mature form of the protein, was reduced in a time-dependent manner. As reported in Fig. 7D, a strong reduction of about 70% was clearly evident even after 3 h exposure to dDAVP and increased at later time points (6 and 12 h). At 24 h of stimulation with dDAVP the mature form of the receptor was undetectable while the intensity of the lower molecular mass bands, likely representing degradation products of AVPR2, was greatly increased, thus suggesting complete degradation of AVPR2.

These finding indicates that β_3AR was resistant to degradation up to 12 h of continuous exposure to the agonist.

β_3AR activation promotes apical AQP2 expression in renal epithelial cells

In renal collecting duct cells, cAMP levels are fundamental regulators of AQP2 translocation to the apical plasma membrane [46]. Therefore, the fact that β_3AR chronic stimulation sustains persistent high levels of cytosolic cAMP should be taken into account when considering AQP2 translocation. Thus, we investigated the effect of long-term β_3AR activation on AQP2 trafficking in MCD4 cells, a clone of M-1 cells that stably express human-AQP2 [50]. These cells were stably transfected with the cDNA that encodes either β_3AR-mCh or AVPR2-mCh.

Fig. 8A reports the confocal analysis of AQP2 localization in MCD4-β_3AR-mCh resting cells after treatment with mirabegron for 1, 3, 6, 12 hours. The cAMP-elevating agent forskolin (FK) was used as positive control for its ability to promote AQP2 exocytosis. AQP2 staining was imaged in a xy confocal plan, passing through the cell apical membrane (upper panel) and in the xz confocal plan. In unstimulated MCD4-β_3AR-mCh cells (cycloheximide alone), most of the AQP2 was located in intracellular and sub-apical vesicles. 1 h mirabegron treatment

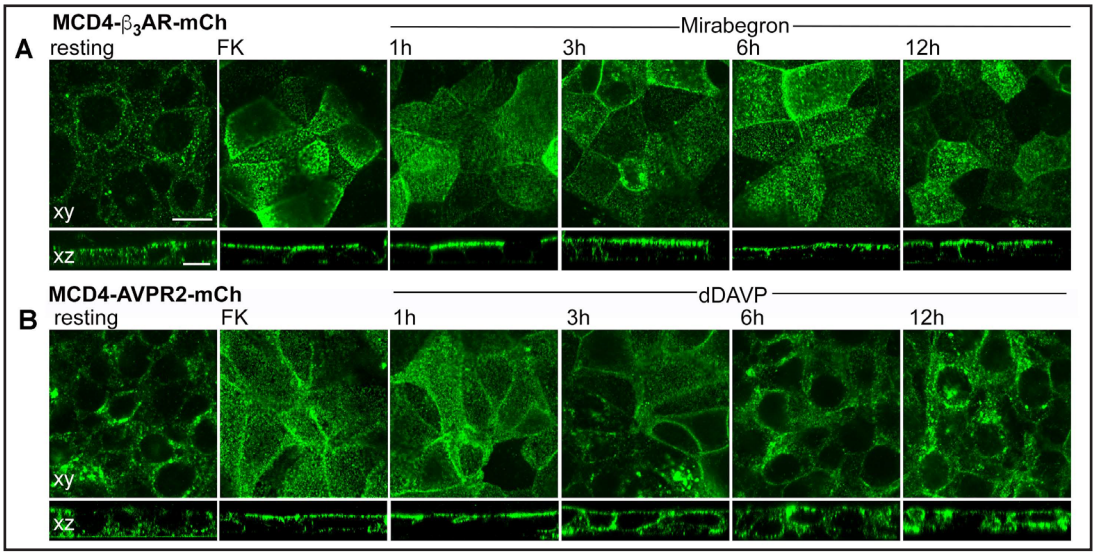

Fig. 8. β_3AR stimulation promotes apical AQP2 expression in renal epithelial cells. Confocal immunofluorescence analysis of AQP2 subcellular localization in MCD4 renal cells stably transfected with β_3AR (MCD4-β_3AR-mCh) or AVPR2 (MCD4-AVPR2-mCh) in resting condition, after 100 μM FK stimulation, and after incubation with 10 nM mirabegron or 100 nM dDAVP, respectively. AQP2 immunostaining was visualized in the xy apical confocal plan (upper panels) and in the xz confocal plan (lower panels). A) Compared to resting conditions, mirabegron, like FK, redistributed AQP2 to the apical plasma membrane. AQP2 translocation induced by mirabegron was clearly evident also after 12 h of continuous stimulation. B) The effect of dDAVP on AQP2 apical expression occurred at 1 and 3 h but at 6 and 12 h AQP2 was localized in intracellular vesicles. Pictures are representative of at least three independent experiments. Scale bars, 20 μm.

resulted in AQP2 redistribution to the apical plasma membrane similar to that obtained by maximal FK stimulation. Interestingly, even at 12 hours of continuous stimulation of β_3AR with mirabegron AQP2 was still exposed at the apical plasma membrane clearly indicating that β_3AR intracellular signaling is not switched off. These results were consistent with our findings on persistent increase of intracellular cAMP, slow internalization and delayed downregulation of β_3AR in M-1 cells.

On the contrary, the effect of dDAVP on AQP2 translocation in MCD4-AVPR2-mCh cells was observed within 3 h (Fig. 8B). As described previously in this study, this evidence was due to a short-lasting cAMP signaling, rapid internalization and downregulation of AVPR2 which already occurred at 3 hours of dDAVP stimulation.

Discussion

Maintaining water homeostasis lies in the ability of the kidneys to concentrate urine and can be mainly attributed to the AVP/AVPR2/AQP2 axis [51]. Under physiological conditions, excessive loss of water through the urine is prevented by the AVP which promotes the osmotic reabsorption of water in the collecting ducts. In kidney collecting duct principal cells, the binding of AVP to basolateral AVPR2 triggers the activation of adenylyl cyclase via Gαs protein, with generation of cAMP that in turn activates protein kinase A (PKA). Consequently, the AQP2 is phosphorylated and translocated from a pool of intracellular storage vesicles to the apical plasma membrane of the principal cells, where it highly increases membrane permeability promoting water reabsorption [52]. This process is reversed by a reduction in circulating AVP levels, which reflects the establishment of isotonicity.

Inactivating mutations of AVPR2 cause X-linked Nephrogenic Diabetes Insipidus (X-NDI). The lack of functional AVPR2 impairs both AQP2 trafficking to the plasma membrane and its transcription in X-NDI patients [53]. Therefore, the kidney loses its ability to concentrate urine leading to the production of large volumes of dilute urine.

Several studies suggested the possibility to restore proper water homeostasis in X-NDI by activating other Gs-coupled receptors expressed in the same renal cells expressing defective AVPR2 [5, 46, 54-56]. However, the potential use of the GPCR exploited so far, such as secretin [46], calcitonin [54] and EP4 [55, 56], is limited by their rapid desensitization [57-59] .

In this respect, β_3AR seems to be a promising drug target to promote antidiuresis bypassing AVPR2 signaling. β_3AR is localized in the AVP-sensitive nephron segments and its activation by a single intraperitoneal injection of BRL3744 induces antidiuresis in mice lacking AVPR2 [5]. Since X-NDI is a congenital condition, long-term treatment with β_3AR agonists may be considered as one of therapeutic possibilities. Thus, as a pre-requisite to predict the feasibility of future clinical trials, we first evaluated *in vitro* whether β_3AR is subject to desensitization upon chronic stimulation in renal cells.

Here, long-term desensitization was investigated with respect to receptor downstream signaling, internalization and degradation.

We first evaluated the signaling pathway through which β_3AR signals in renal cells. In the present study, we showed that mirabegron-induced β_3AR activation, elevated intracellular cAMP and activates PKA. These results are consistent with our recent study in which, in mouse kidney tubule suspensions, treatment with specific agonist for mouse β_3AR led to a concentration-dependent increase in intracellular cAMP [5]. In addition, we show that mirabegron induced not synchronous Ca^{2+} transients in kidney cells expressing β_3AR. These results are in line with previous reports on the intracellular Ca^{2+} mobilization triggered by cAMP-mediated agonists, including AVP and dDAVP [60-63]. Furthermore, previous studies pointed out a key role for localized increases in intracellular Ca^{2+} concentration as trigger for the fusion of vesicles to their target membranes [16], as also reported for the AVP-dependent exocytotic insertion of AQP2 [63]. Thus, mirabegron-induced Ca^{2+} increases might be important for the fusion of AQP2 vesicles to the apical membranes.

In the present study, β_3AR showed preserved cAMP signaling even after 12 h of continuous agonist exposure. Different studies indicated that long-term activation of β_3AR (up to 24 h) induced adenylyl cyclase activity [14, 20] and cAMP signaling [19] that were, however, significantly reduced over the time course of the experiments. Considering the small amount of receptor internalization, the sustained increase in cAMP level under chronic activation of β_3AR is likely due to the functional receptor localized at the basolateral membrane and not within the endocytic pathway, as reported for other GPCRs [48, 64, 65]. In addition, the small reduction in the expression level of β_3AR after 6 h probably indicates that internalized β_3AR undergoes degradation. Previous independent studies showed that, in chinese hamster fibroblasts (CHW) cells expressing β_3AR [17, 66], the receptor density on the plasma membrane was unchanged after 24 h treatment with isoproterenol. However, both studies were performed in the absence of cycloheximide, thus membrane insertion of newly synthesized receptor cannot be excluded. However, these evidences seemed strictly dependent by the cell type since, under the same chronic stimulation, β_3AR was significantly reduced in murine L fibroblast cells and human neuroblastoma SK-N-MC cells [66, 67].

Overall, these findings strongly suggested that, at least *in vitro,* human β_3AR is resistant to chronic agonist-induced desensitization in renal cells. Of note, this effect is not an artifact due to the presence of the large mCherry tag, since same results were also obtained with the small FLAG® tag (see Fig. 3B and 5).

In addition, we demonstrated that long-term β_3AR stimulation with mirabegron, leading to persistent higher levels of cytosolic cAMP, keeps promoting AQP2 apical membrane expression up to 12 h in renal cells. The persistent effect on the AQP2 apical localization likely supports the hypothesis that chronic β_3AR agonist use could be exploited to promote water reabsorption for the long time *in vivo*. It should be noted that β_3AR agonists are already approved by the American food and drug administration (FDA) and the European Medicines Agency (EMA) for the treatment of overactive bladder (OAB) and mirabegron is the first drug licensed for this use [68, 69]. It is a safe, effective and well-tolerated new class of drug [70-73]. Considering the expression of β_3AR in the heart, it has been demonstrated, by several studies, that mirabegron does not cause clinically relevant changes in cardiovascular safety [73].

In our study, parallel experiments regarding AVPR2 highlighted the substantial desensitization differences between AVPR2, selected by the evolution as the main mediator of the antidiuretic action in the kidney and β_3AR, an alternative GPCR proposed to mimic AVPR2 effects.

We show here that AVPR2 was largely sequestrated within intracellular vesicles and strongly downregulated with no significant downstream signaling after 3 h exposure to dDAVP. In fact, physiological effects of AVPR2 are very rapid and promptly reversible [74]. Vasopressin has a very short biological half-life, about 3 min [74], and exhibit robust circadian oscillations increasing osmolality and decreasing volume of the urine during sleep to prevents nocturnal polyuria and sleep disruptions [75]. These mechanisms are likely preserved in X-NDI patients, although the absence of functional AVPR2 prevents a proper antidiuresis. Therefore, stimulation of another GPCR expressed in kidney principal cells, might circumvent the AVPR2 defect. Of note, β_3AR is less prone than AVPR2 to agonist-induced desensitization in renal collecting duct epithelial cells and its chronic stimulation promoted a continuous effect on AQP2 apical expression. These findings encourage preclinical studies supporting the hypothesis that chronic β_3AR stimulation should promote effective antidiuresis in X-NDI patients.

Disclosure Statement

This work was supported by the Fondazione Telethon (project # GGP15083) to M.S. The authors declare to have no conflict.

References

1 Tan S, Curtis-Prior PB: Characterization of the beta-adrenoceptor of the adipose cell of the rat. Int J Obes 1983;7:409-414.
2 Arch JR, Ainsworth AT, Cawthorne MA, Piercy V, Sennitt MV, Thody VE, Wilson C, Wilson S: Atypical beta-adrenoceptor on brown adipocytes as target for anti-obesity drugs. Nature 1984;309:163-165.
3 Emorine LJ, Marullo S, Briend-Sutren MM, Patey G, Tate K, Delavier-Klutchko C, Strosberg AD: Molecular characterization of the human beta 3-adrenergic receptor. Science 1989;245:1118-1121.
4 Ursino MG, Vasina V, Raschi E, Crema F, De Ponti F: The beta3-adrenoceptor as a therapeutic target: current perspectives. Pharmacol Res 2009;59:221-234.
5 Procino G, Carmosino M, Milano S, Dal Monte M, Schena G, Mastrodonato M, Gerbino A, Bagnoli P, Svelto M: beta3 adrenergic receptor in the kidney may be a new player in sympathetic regulation of renal function. Kidney Int 2016;90:555-567.
6 Bockenhauer D, Bichet DG: Pathophysiology, diagnosis and management of nephrogenic diabetes insipidus. Nat Rev Nephrol 2015;11:576-588.
7 Wesche D, Deen PM, Knoers NV: Congenital nephrogenic diabetes insipidus: the current state of affairs. Pediatr Nephrol 2012;27:2183-2204.
8 Giuseppe Procino SMMCAGLBPP, Maria S: Hereditary Nephrogenic Diabetes Insipidus: Molecular Basis of the Defect and Potential Novel Strategies for Treatment. J Genet Syndrom Gen Therap 2014;5:1-14.
9 Milano S, Carmosino M, Gerbino A, Svelto M, Procino G: Hereditary Nephrogenic Diabetes Insipidus: Pathophysiology and Possible Treatment. An Update. Int J Mol Sci 2017;18
10 Hausdorff WP, Caron MG, Lefkowitz RJ: Turning off the signal: desensitization of beta-adrenergic receptor function. FASEB J 1990;4:2881-2889.
11 Bohm SK, Grady EF, Bunnett NW: Regulatory mechanisms that modulate signalling by G-protein-coupled receptors. Biochem J 1997;322:1-18.
12 Grady EF, Bohm SK, Bunnett NW: Turning off the signal: mechanisms that attenuate signaling by G protein-coupled receptors. Am J Physiol 1997;273:G586-601.
13 Carpene C, Galitzky J, Collon P, Esclapez F, Dauzats M, Lafontan M: Desensitization of beta-1 and beta-2, but not beta-3, adrenoceptor-mediated lipolytic responses of adipocytes after long-term norepinephrine infusion. J Pharmacol Exp Ther 1993;265:237-247.
14 Chaudhry A, Granneman JG: Influence of cell type upon the desensitization of the beta 3-adrenergic receptor. J Pharmacol Exp Ther 1994;271:1253-1258.
15 Thomas RF, Holt BD, Schwinn DA, Liggett SB: Long-term agonist exposure induces upregulation of beta 3-adrenergic receptor expression via multiple cAMP response elements. Proc Natl Acad Sci U S A 1992;89:4490-4494.
16 Curran PK, Fishman PH: Endogenous β3- but not β1-adrenergic receptors are resistant to agonist-mediated regulation in human SK-N-MC neurotumor cells. Cell Signal 1996;8:355-364.
17 Liggett SB, Freedman NJ, Schwinn DA, Lefkowitz RJ: Structural basis for receptor subtype-specific regulation revealed by a chimeric beta 3/beta 2-adrenergic receptor. Proc Natl Acad Sci U S A 1993;90:3665-3669.
18 Nantel F, Bonin H, Emorine LJ, Zilberfarb V, Strosberg AD, Bouvier M, Marullo S: The human beta 3-adrenergic receptor is resistant to short term agonist-promoted desensitization. Mol Pharmacol 1993;43:548-555.
19 Candelore MR, Deng L, Tota LM, Kelly LJ, Cascieri MA, Strader CD: Pharmacological characterization of a recently described human beta 3-adrenergic receptor mutant. Endocrinology 1996;137:2638-2641.
20 Chambers J, Park J, Cronk D, Chapman C, Kennedy FR, Wilson S, Milligan G: Beta 3-adrenoceptor agonist-induced down-regulation of Gs alpha and functional desensitization in a Chinese hamster ovary cell line expressing a beta 3-adrenoceptor refractory to down-regulation. Biochem J 1994;303 (Pt 3):973-978.
21 Nantel F, Bouvier M, Strosberg AD, Marullo S: Functional effects of long-term activation on human beta 2- and beta 3-adrenoceptor signalling. Br J Pharmacol 1995;114:1045-1051.
22 Bengtsson T, Redegren K, Strosberg AD, Nedergaard J, Cannon B: Down-regulation of beta3 adrenoreceptor gene expression in brown fat cells is transient and recovery is dependent upon a short-lived protein factor. J Biol Chem 1996;271:33366-33375.

23 Granneman JG, Lahners KN: Differential adrenergic regulation of beta 1- and beta 3-adrenoreceptor messenger ribonucleic acids in adipose tissues. Endocrinology 1992;130:109-114.
24 Hutchinson DS, Evans BA, Summers RJ: beta(3)-adrenoceptor regulation and relaxation responses in mouse ileum. Br J Pharmacol 2000;129:1251-1259.
25 Scarpace PJ, Matheny M, Tumer N: Differential down-regulation of beta3-adrenergic receptor mRNA and signal transduction by cold exposure in brown adipose tissue of young and senescent rats. Pflugers Arch 1999;437:479-483.
26 Vrydag W, Alewijnse AE, Michel MC: Do gene polymorphisms alone or in combination affect the function of human beta3-adrenoceptors? Br J Pharmacol 2009;156:127-134.
27 Seifert R: A door opener for future research: agonist-induced beta3-adrenoceptor desensitization in HEK cells but not CHO cells. Naunyn Schmiedebergs Arch Pharmacol 2013;386:841-842.
28 Sacco E, Bientinesi R: Mirabegron: a review of recent data and its prospects in the management of overactive bladder. Ther Adv Urol 2012;4:315-324.
29 Igawa Y, Michel MC: Pharmacological profile of beta3-adrenoceptor agonists in clinical development for the treatment of overactive bladder syndrome. Naunyn Schmiedebergs Arch Pharmacol 2013;386:177-183.
30 Stoos BA, Naray-Fejes-Toth A, Carretero OA, Ito S, Fejes-Toth G: Characterization of a mouse cortical collecting duct cell line. Kidney Int 1991;39:1168-1175.
31 Bouley R, Lin HY, Raychowdhury MK, Marshansky V, Brown D, Ausiello DA: Downregulation of the vasopressin type 2 receptor after vasopressin-induced internalization: involvement of a lysosomal degradation pathway. Am J Physiol Cell Physiol 2005;288:C1390-1401.
32 Innamorati G, Sadeghi H, Eberle AN, Birnbaumer M: Phosphorylation of the V2 vasopressin receptor. The J Biol Chem 1997;272:2486-2492.
33 Martin NP, Lefkowitz RJ, Shenoy SK: Regulation of V2 vasopressin receptor degradation by agonist-promoted ubiquitination. J Biol Chem 2003;278:45954-45959.
34 Robben JH, Knoers NV, Deen PM: Regulation of the vasopressin V2 receptor by vasopressin in polarized renal collecting duct cells. Mol Biol Cell 2004;15:5693-5699.
35 Tamma G, Procino G, Strafino A, Bononi E, Meyer G, Paulmichl M, Formoso V, Svelto M, Valenti G: Hypotonicity induces aquaporin-2 internalization and cytosol-to-membrane translocation of ICln in renal cells. Endocrinology 2007;148:1118-1130.
36 Procino G, Barbieri C, Tamma G, De Benedictis L, Pessin JE, Svelto M, Valenti G: AQP2 exocytosis in the renal collecting duct -- involvement of SNARE isoforms and the regulatory role of Munc18b. J Cell Sci 2008;121:2097-2106.
37 Iolascon A, Aglio V, Tamma G, D'Apolito M, Addabbo F, Procino G, Simonetti MC, Montini G, Gesualdo L, Debler EW, Svelto M, Valenti G: Characterization of two novel missense mutations in the AQP2 gene causing nephrogenic diabetes insipidus. Nephron Physiology 2007;105:p33-41.
38 van der Krogt GN, Ogink J, Ponsioen B, Jalink K: A comparison of donor-acceptor pairs for genetically encoded FRET sensors: application to the Epac cAMP sensor as an example. PLoS One 2008;3:e1916.
39 Depry C, Allen MD, Zhang J: Visualization of PKA activity in plasma membrane microdomains. Mol Biosyst 2011;7:52-58.
40 Lefkimmiatis K, Moyer MP, Curci S, Hofer AM: "cAMP sponge": a buffer for cyclic adenosine 3', 5'-monophosphate. PLoS One 2009;4:e7649.
41 Procino G, Gerbino, A., Milano, S., Nicoletti, M. C., Mastrofrancesco, L., Carmosino, M., & Svelto, M.: Rosiglitazone Promotes AQP2 Plasma Membrane Expression In Renal Cells Via a Ca2+-Dependent/cAMP-Indipendent Mechanism. Cell Physiol Biochem 2015;35:15.
42 Post SR, Rump LC, Zambon A, Hughes RJ, Buda MD, Jacobson JP, Kao CC, Insel PA: ATP activates cAMP production via multiple purinergic receptors in MDCK-D1 epithelial cells. Blockade of an autocrine/paracrine pathway to define receptor preference of an agonist. J Biol Chem 1998;273:23093-23097.
43 Deen PM, Rijss JP, Mulders SM, Errington RJ, van Baal J, van Os CH: Aquaporin-2 transfection of Madin-Darby canine kidney cells reconstitutes vasopressin-regulated transcellular osmotic water transport. JASN 1997;8:1493-1501.
44 Gerbino A, Schena G, Milano S, Milella L, Barbosa AF, Armentano F, Procino G, Svelto M, Carmosino M: Spilanthol from Acmella Oleracea Lowers the Intracellular Levels of cAMP Impairing NKCC2 Phosphorylation and Water Channel AQP2 Membrane Expression in Mouse Kidney. PLoS One 2016;11:e0156021.

45 Carmosino M, Gerbino A, Hendy GN, Torretta S, Rizzo F, Debellis L, Procino G, Svelto M: NKCC2 activity is inhibited by the Bartter's syndrome type 5 gain-of-function CaR-A843E mutant in renal cells. Biol Cell 2015;107:98-110.
46 Procino G, Milano S, Carmosino M, Barbieri C, Nicoletti MC, Li JH, Wess J, Svelto M: Combination of secretin and fluvastatin ameliorates the polyuria associated with X-linked nephrogenic diabetes insipidus in mice. Kidney Int 2014;86:127-138.
47 Burdyga A, Lefkimmiatis K: Simultaneous assessment of cAMP signaling events in different cellular compartments using FRET-based reporters. Methods Mol Biol 2015;1294:1-12.
48 Feinstein TN, Yui N, Webber MJ, Wehbi VL, Stevenson HP, King JD, Jr., Hallows KR, Brown D, Bouley R, Vilardaga JP: Noncanonical control of vasopressin receptor type 2 signaling by retromer and arrestin. J Biol Chem 2013;288:27849-27860.
49 Innamorati G, Le Gouill C, Balamotis M, Birnbaumer M: The long and the short cycle. Alternative intracellular routes for trafficking of G-protein-coupled receptors. J Biol Chem 2001;276:13096-13103.
50 Iolascon A, Aglio V, Tamma G, D'Apolito M, Addabbo F, Procino G, Simonetti MC, Montini G, Gesualdo L, Debler EW, Svelto M, Valenti G: Characterization of two novel missense mutations in the AQP2 gene causing nephrogenic diabetes insipidus. Nephron Physiol 2007;105:p33-41.
51 Juul KV: The evolutionary origin of the vasopressin/V2-type receptor/aquaporin axis and the urine-concentrating mechanism. Endocrine 2012;42:63-68.
52 Fushimi K, Sasaki S, Marumo F: Phosphorylation of serine 256 is required for cAMP-dependent regulatory exocytosis of the aquaporin-2 water channel. J biol chem 1997;272:14800-14804.
53 Matsumura Y, Uchida S, Rai T, Sasaki S, Marumo F: Transcriptional regulation of aquaporin-2 water channel gene by cAMP. J Am Soc Nephrol 1997;8:861-867.
54 Bouley R, Lu HA, Nunes P, Da Silva N, McLaughlin M, Chen Y, Brown D: Calcitonin has a vasopressin-like effect on aquaporin-2 trafficking and urinary concentration. J Am Soc Nephrol 2011;22:59-72.
55 Li JH, Chou CL, Li B, Gavrilova O, Eisner C, Schnermann J, Anderson SA, Deng CX, Knepper MA, Wess J: A selective EP4 PGE2 receptor agonist alleviates disease in a new mouse model of X-linked nephrogenic diabetes insipidus. J Clin Invest 2009;119:3115-3126.
56 Olesen ET, Rutzler MR, Moeller HB, Praetorius HA, Fenton RA: Vasopressin-independent targeting of aquaporin-2 by selective E-prostanoid receptor agonists alleviates nephrogenic diabetes insipidus. Proc Natl Acad Sci U S A 2011;108:12949-12954.
57 Ozcelebi F, Holtmann MH, Rentsch RU, Rao R, Miller LJ: Agonist-stimulated phosphorylation of the carboxyl-terminal tail of the secretin receptor. Mol Pharmacol 1995;48:818-824.
58 Samura A, Wada S, Suda S, Iitaka M, Katayama S: Calcitonin receptor regulation and responsiveness to calcitonin in human osteoclast-like cells prepared *in vitro* using receptor activator of nuclear factor-kappaB ligand and macrophage colony-stimulating factor. Endocrinology 2000;141:3774-3782.
59 Nishigaki N, Negishi M, Ichikawa A: Two Gs-coupled prostaglandin E receptor subtypes, EP2 and EP4, differ in desensitization and sensitivity to the metabolic inactivation of the agonist. Mol Pharmacol 1996;50:1031-1037.
60 Chou CL, Yip KP, Michea L, Kador K, Ferraris JD, Wade JB, Knepper MA: Regulation of aquaporin-2 trafficking by vasopressin in the renal collecting duct. Roles of ryanodine-sensitive Ca2+ stores and calmodulin. J Biol Chem 2000;275:36839-36846.
61 Ecelbarger CA, Chou CL, Lolait SJ, Knepper MA, DiGiovanni SR: Evidence for dual signaling pathways for V2 vasopressin receptor in rat inner medullary collecting duct. Am J Physiol 1996;270:F623-633.
62 Yip KP: Coupling of vasopressin-induced intracellular Ca2+ mobilization and apical exocytosis in perfused rat kidney collecting duct. J Physiol 2002;538:891-899.
63 Balasubramanian L, Sham JS, Yip KP: Calcium signaling in vasopressin-induced aquaporin-2 trafficking. Pflugers Arch 2008;456:747-754.
64 Feinstein TN, Wehbi VL, Ardura JA, Wheeler DS, Ferrandon S, Gardella TJ, Vilardaga JP: Retromer terminates the generation of cAMP by internalized PTH receptors. Nat Chem Biol 2011;7:278-284.
65 Calebiro D, Nikolaev VO, Gagliani MC, de Filippis T, Dees C, Tacchetti C, Persani L, Lohse MJ: Persistent cAMP-signals triggered by internalized G-protein-coupled receptors. PLoS Biol 2009;7:e1000172.
66 Nantel F, Marullo S, Krief S, Strosberg AD, Bouvier M: Cell-specific down-regulation of the beta 3-adrenergic receptor. J Biol Chem 1994;269:13148-13155.

67 Esbenshade TA, Han C, Theroux TL, Granneman JG, Minneman KP: Coexisting beta 1- and atypical beta-adrenergic receptors cause redundant increases in cyclic AMP in human neuroblastoma cells. Mol Pharmacol 1992;42:753-759.
68 Abrams P, Kelleher C, Staskin D, Rechberger T, Kay R, Martina R, Newgreen D, Paireddy A, van Maanen R, Ridder A: Combination treatment with mirabegron and solifenacin in patients with overactive bladder: efficacy and safety results from a randomised, double-blind, dose-ranging, phase 2 study (Symphony). Eur Urol 2015;67:577-588.
69 Warren K, Burden H, Abrams P: Mirabegron in overactive bladder patients: efficacy review and update on drug safety. Ther Adv Drug Saf 2016;7:204-216.
70 Herschorn S, Barkin J, Castro-Diaz D, Frankel JM, Espuna-Pons M, Gousse AE, Stolzel M, Martin N, Gunther A, Van Kerrebroeck P: A phase III, randomized, double-blind, parallel-group, placebo-controlled, multicentre study to assess the efficacy and safety of the beta(3) adrenoceptor agonist, mirabegron, in patients with symptoms of overactive bladder. Urology 2013;82:313-320.
71 Nitti VW, Auerbach S, Martin N, Calhoun A, Lee M, Herschorn S: Results of a randomized phase III trial of mirabegron in patients with overactive bladder. J Urol 2013;189:1388-1395.
72 Yamaguchi O, Marui E, Kakizaki H, Homma Y, Igawa Y, Takeda M, Nishizawa O, Gotoh M, Yoshida M, Yokoyama O, Seki N, Ikeda Y, Ohkawa S: Phase III, randomised, double-blind, placebo-controlled study of the beta3-adrenoceptor agonist mirabegron, 50 mg once daily, in Japanese patients with overactive bladder. BJU Int 2014;113:951-960.
73 Chapple CR, Cardozo L, Nitti VW, Siddiqui E, Michel MC: Mirabegron in overactive bladder: a review of efficacy, safety, and tolerability. Neurourol Urodyn 2014;33:17-30.
74 Bankir L, Bichet DG, Morgenthaler NG: Vasopressin: physiology, assessment and osmosensation. J Intern Med 2017;282:284-297.
75 Firsov D, Bonny O: Circadian regulation of renal function. Kidney Int 2010;78:640-645