Molecular and Cellular Endocrinology 441 (2017) 99–107

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

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

Role of cAMP/PKA pathway and T-type calcium channels in the mechanism of action of serotonin in human adrenocortical cells

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ARTICLE INFO

Article history:
Received 13 July 2016
Received in revised form
30 September 2016
Accepted 9 October 2016
Available online 12 October 2016

Keywords:
5-HT
Cortisol
Calcium current
cAMP
Adrenocortical cells
Protein kinase A

ABSTRACT

In human adrenal, serotonin (5-HT), produced by mast cells located in zona glomerulosa, stimulates production of corticosteroids through a paracrine mechanism involving the 5-HT receptor type 4 (5-HT_4). The aim of the present study was to investigate the transduction mechanisms associated with activation of 5-HT_4 receptors in human adrenocortical cells. Our results show that 5-HT_4 receptors are present in the outer adrenal cortex, both in glomerulosa and fasciculata zonae. In the zona glomerulosa. 5-HT_4 receptor was detected both in immunopositive and immunonegative cells for 11β-hydroxylase, an enzyme involved in cortisol synthesis. The data demonstrate that 5-HT_4 receptors are positively coupled to adenylyl cyclases and cAMP-dependent protein kinases (PKA). The activation of the cAMP-PKA pathway is associated with calcium influx through T-type calcium channels. Both the adenylyl cyclase/PKA pathway and the calcium influx are involved in 5-HT-induced cortisol secretion.

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1. Introduction

The adrenal cortex participates in sodium and glucose homeostasis through secretion of aldosterone and cortisol (costicosterone in rodents). Aldosterone synthesis is determined by aldosterone synthase, an enzyme encoded by *CYP11B2*, while cortisol production is dependent on expression of 17α- and 11β-hydroxylases encoded by *CYP17A1* and *CYP11B1*, respectively (Schiffer et al., 2015). In adult human adrenal, expression of *CYP11B2* is restricted to aldosterone-producing cell clusters (APCC) present in the zona glomerulosa, whereas *CYP11B1* is expressed by adrenocortical cells localized in zona fasciculata and in areas of zona glomerulosa outside APCC (Gomez-Sanchez et al., 2014; Nishimoto et al., 2010; Vinson, 2016), indicating that aldosterone is provided by a subpopulation of zona glomerulosa cells and cortisol synthesis may occur in both glomerulosa and fasciculata zonae.

Aldosterone secretion is under control of plasma potassium and the renin angiotensin system (Bollag, 2014; Hattangady et al., 2012). High plasma K^+ concentration provokes membrane depolarization which activates voltage-dependent Ca^{2+} channels generating Ca^{2+} influx. Angiotensin receptors are positively coupled to the phospholipase C (PLC)-inositol trisphosphate (IP_3)-Ca^{2+}/calmodulin-dependent kinase II (CaMKII) pathway. Cortisol production is regulated by the adrenocorticotropic hormone ACTH acting on melanocortin type 2 receptors (MC2R) coupled to the cAMP/protein kinase A (PKA) pathway (Gallo-Payet, 2016). Corticosteroid production is also highly dependent on variation of cytosolic calcium induced by these regulatory factors (Barrett et al., 2016; Rossier, 2016; Spät et al., 2016). Ca^{2+} signaling results from IP_3-induced Ca^{2+} release from intracellular pools and/or calcium influx through voltage-sensitive membrane channels.

Abbreviations: 5-HT, 5-hydroxytryptamine, serotonin; 5-HT_4, 5-HT receptor type 4; ACTH, adrenocorticotropic hormone; MC2R, melanocortin type 2 receptor; PKA, protein kinase A; APCC, aldosterone-producing cell clusters; PLC, phospholipase C; IP_3, inositol trisphosphate; CaMKII, Ca^{2+}/calmodulin-dependent kinase II; cAMP, adenosine 3′,5′-cyclic monophosphate; PKA, protein kinase A; dbcAMP, dibutyryladenosine 3′,5′-cyclic monophosphate; IBMX, 1-methyl-3-isobutylxanthine; ($[Ca^{2+}]_i$, intracellular calcium concentration.

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http://dx.doi.org/10.1016/j.mce.2016.10.008
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Besides circulating regulators, different intraadrenal factors modulate secretion of aldosterone and cortisol (Ehrhart-Bornstein et al., 1998; Lefebvre et al., 2013). In particular, 5-HT, produced by mast cells located in zona glomerulosa, is able to stimulate production of corticosteroids through a paracrine mechanism involving the 5-HT receptor type 4 (5-HT_4) expressed by adrenocortical cells (Contesse et al., 2002; Lefebvre et al., 2001, 1993, 1992). Moreover, overexpression of 5-HT_4 receptors has been reported in adrenal tumors responsible for hyperaldosteronism and hypercortisolism (Cushing's syndrome) (Bertherat et al., 2005; Cartier et al., 2005; El Ghorayeb et al., 2015; Lefebvre et al., 2002). Deciphering the mechanism of action of 5-HT_4 receptors in adrenal is critical to understand the role of the serotonergic control of corticosteroid secretion in physiological and pathological conditions. Positive coupling of 5-HT_4 receptors to adenylyl cyclase and calcium influx has been well documented in the central nervous system, gut and heart (Castro et al., 2005; Huang and Kandel, 2007; Lee et al., 2014; Robert et al., 2001; Weninger et al., 2014). In the present study, we investigated the localization of 5-HT_4 receptors in human adrenal cortex and their second messenger systems including the cAMP/PKA pathway and membrane calcium currents.

2. Materials and methods

2.1. Tissue collection

The adrenal glands were collected from 37 patients undergoing expanded nephrectomy for kidney cancer. Tissue explants were obtained at surgery and immediately dissected by the pathologist. Specimens for immunohistofluorescence study (n = 3) were formalin-fixed, embedded in paraffin and then cut into 5 μm sections. Specimens for functional studies (n = 34) were immersed in DMEM supplemented with 1% antibiotic–antimycotic solution composed of 10 000 unit/ml penicillin, 10 000 μg/ml streptomycin and 25 μg/ml amphotericin B (Fisher Scientific, Illkirch, France) and transported to the laboratory. The protocol of collection of the tissues and the experimental procedures were approved by the regional ethics committees, and informed consent was obtained from all subjects.

2.2. Test substances

Serotonin, dibutyryladenosine 3′,5′-cyclic monophosphate (dbcAMP), 1-methyl-3-isobutylxanthine (IBMX), chelerythrine, H-89, GR113808, zacopride, EGTA, nifedipine, mibefradil, $NiCl_2$ were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). ω-conotoxin and sFTX-3.3 were obtained from Alomone Labs (Jerusalem, Israel).

2.3. Immunofluorescence studies

The tissue sections were deparaffinized and re-hydrated. Antigen retrieval was performed in a Tris-EDTA buffer (pH 9.0) added with 0.05% sodium dodecyl sulfate (SDS) at 95 C for 30 min. The sections were incubated with 10% normal donkey serum (NDS), 0.5% SDS in 0.05 M Tris-buffered saline at room temperature for 30 min. They were then incubated overnight at 4 C in a humidified atmosphere with rabbit polyclonal 5-HT_4 receptor antibody (LS-A2685; LifeSpan Bio) in combination with rat monoclonal anti-hCYP11B1 (1:50) in 0.05 M Tris-buffered saline, 10% NDS, 0.2% Tween20. The sections were washed and then incubated for 90 min with secondary antibodies donkey anti-rabbit IgG conjugated to Alexa 488 (1:300) and donkey anti-rabbit IgG conjugated to Alexa 594 (1:300) (Life Technologies). The sections were incubated with DAPI (1 mg/L) and then 0.5% Sudan black B in 70% ethanol to reduce autofluorescence. Coverslips were mounted using mounting medium (Dako). Images were acquired using a confocal laser scanning microscope (TCS SP8 MP; Leica Microsystems, Reuil-Malmaison, France) on PRIMACEN, the Cell Imaging Platform of Normandy, University of Rouen.

2.4. cAMP measurement

Adrenocortical explants were cut into 2- to 3-mm pieces in HBSS (130 mM NaCl, 3.5 mM KCl, 1.8 mM $CaCl_2$, 0.5 mM $MgCl_2$, 2.5 mM $NaHCO_3$, 5 mM HEPES, supplemented with 1 g/liter bovine serum albumin, 1 g/liter glucose, and 1% of antimycotic/antibiotic solution). The tissue fragments were preincubated with 10^{-4} M IBMX for 30 min at 37 C, and then treated with 5-HT in the absence or presence of GR113808 (10^{-6} M) for 30 min. The reaction was stopped by addition of ice-cold 20% trichloroacetic acid. Adrenal fragments were homogenized, washed with water-satured diethyl ether and lyophilized. The concentration of cAMP contained in the dried extract was quantified by radioimmunoassay (Institute of isotopes, Budapest).

2.5. Cell culture

Tissue samples were stirred for 45 min at 37 C in culture medium containing collagenase type 1A (60 mg/ml; Sigma–Aldrich) and desoxyribonuclease 1 type 4 (4 mg/ml; Sigma–Aldrich) in a 5% CO2–95% air atmosphere. Dispersed cells were suspended in culture medium (50% DMEM, 50% Ham–F12; Life Technologies, Thermo Scientific, Villebon-sur-Yvette, France) supplemented with 1% insulin–transferrin–selenium solution (Gibco, Thermo Scientific) and 5% fetal calf serum (Sigma-Aldrich). Cells were plated at a density of 10^6 cells/ml on 24-well culture dishes or poly-lysine coated coverslips. Adrenocortical cells were cultured at 37 C in a 5% CO2–95% air atmosphere with 100% relative humidity. The culture medium was changed 24 h after plating to remove non-adherent chromaffin cells.

2.6. Incubation for cortisol secretion measurement

Incubation experiments were conducted in quadruplicate on 2-days cultured adrenocortical cells. Cells were incubated for 24 h at 37 C with fresh DMEM (control experiments) or DMEM with 5-HT in the absence or presence of different test substances. Then, incubation medium was immediately frozen until cortisol assay.

2.7. Perifusion experiments

Fragments of adrenocortical explants (2–3 mm) were mixed with biogel P2 and transferred into perifusion chambers, as previously described (Lefebvre et al., 1992). The tissue fragments were perifused with DMEM at constant flow rate (300 μl/min). The perifusion medium was continuously gassed with a 95% O2–5% CO_2 mixture and maintained at 37 C. The tissues were allowed to stabilize for 2 h before any test substance was administered. Test substances were dissolved in gassed DMEM and infused into the perifusion chambers at the same flow rate as DMEM alone, by means of a multichannel peristaltic pump. Fractions of the effluent perifusate were collected every 5 min and immediately frozen until cortisol assay.

2.8. Cortisol radioimmunoassay

Cortisol secretion was quantified by radioimmunoassay procedure using specific antibodies (Lefebvre et al., 1992) and tritiated

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