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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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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-HT₄). The aim of the present study was to investigate the transduction mechanisms associated with activation of 5-HT₄ receptors in human adrenocortical cells. Our results show that 5-HT₄ receptors are present in the outer adrenal cortex, both in glomerulosa and fasciculata zonae. In the zona glomerulosa, 5-HT₄ receptor was detected both in immunopositive and immunonegative cells for 11 β -hydroxylase, an enzyme involved in cortisol synthesis. The data demonstrate that 5-HT₄ 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.

Keywords

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

1. Introduction

The adrenal cortex participates in sodium and glucose homeostasis through secretion of aldosterone and cortisol (corticosterone in rodents). Aldosterone synthesis is determined by aldosterone synthase, an enzyme encoded by *CYP11B2*, while cortisol production is dependent on expression of 17 α - and 11 β -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

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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 sub-population 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 adrenocorticotrophic 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; Spat 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.

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-HT₄) expressed by adrenocortical cells (Contesse et al., 2002; Lefebvre et al., 2001, 1993, 1992). Moreover, overexpression of 5-HT₄ 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-HT₄ 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-HT₄ 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-HT₄ 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 μ m 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 μ g/ml streptomycin and 25 μ g/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, dibutyryladenine 3',5'-cyclic monophosphate (dbcAMP), 1-methyl-3-isobutylxanthine (IBMX), chelerythrine, H-89, GR113808, zacopride, EGTA, nifedipine, mibefradil, NiCl₂ 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-HT₄ 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 CaCl₂, 0.5 mM MgCl₂, 2.5 mM NaHCO₃, 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-saturated 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 10⁶ 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. Perfusion experiments

Fragments of adrenocortical explants (2–3 mm) were mixed with biogel P2 and transferred into perfusion chambers, as previously described (Lefebvre et al., 1992). The tissue fragments were perfused with DMEM at constant flow rate (300 μ l/min). The perfusion medium was continuously gassed with a 95% O₂–5% CO₂ 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 perfusion chambers at the same flow rate as DMEM alone, by means of a multichannel peristaltic pump. Fractions of the effluent perfusate 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 steroid hormones (Perkin Elmer, Villebon-sur-Yvette, France) as previously reported (Tetsi Nomigni et al., 2015). Sensitivity of the assay was 15 pg. Cross-reactivity of cortisol antibodies for progesterone, 17-hydroxyprogesterone, 17-hydroxypregnenolone and aldosterone was less than 0.01%.

2.9. Measurement of cytosolic calcium concentration

Cytosolic calcium concentration measurements were performed on 3- to 5-day-old cultured adrenocortical cells. Briefly, cultured cells were incubated in the dark for 45 min at 37 C with 5×10^{-6} M indo-1/AM (Molecular Probes, Eugene, OR) in DMEM and rinsed twice with fresh medium. The intracellular calcium concentration ($[Ca^{2+}]_i$) was monitored at 37 C using a dual emission micro-fluorimeter system, as previously described (Lenglet et al., 2002). Test substances were applied in the vicinity of the studied cells by a pressure ejection system. The concentrations indicated are those contained in the ejection pipette, which is necessarily much higher than that occurring at the cell surface.

2.10. Recording of calcium currents

Calcium currents were recorded at room temperature on 3- to 5-day-old cultured adrenocortical cells by using the patch-clamp technique in the whole-cell configuration. Patch pipettes (3–5 M Ω) were filled with a solution containing 130 mM CsCl, 20 mM NaCl, 5 mM EGTA, 2 mM ATP, 0.2 mM GTP, and 5 mM HEPES (pH 7.3). Calcium currents were recorded by using an extracellular solution containing 130 mM tetraethylammonium chloride, 10 mM CaCl₂, 5 mM CsCl, 1 mM MgCl₂, and 5 mM HEPES (pH 7.4). The bathing medium was renewed continuously with fresh extracellular solution at a flow rate of 2 ml/min. Test substances were mixed with the extracellular solution and administered for 5 min in the vicinity of the studied cells. The electrical signals were amplified with an

Axopatch 200B (Axon Instruments, Foster City, CA). Holding potential was -80 mV. The currents were filtered at 5 kHz and sampled at 2 kHz with a Digidata 1320 interface and pClamp 8 software (Axon Instruments). Voltage pulses were applied at 0.1 Hz. Leak currents were subtracted by using the P/4 protocol corresponding to series of sub-pulses of opposite polarity included in the pClamp 8 software.

2.11. Statistical analysis

Results are expressed as mean \pm sem. Statistical significance was assessed by Bonferroni's test after one-way ANOVA by using the Prism 4 program (GraphPad Software, Inc.).

3. Results

3.1. Localization of CYP11B1 and 5-HT₄ receptors in human adrenal

5-HT₄ receptor immunoreactivity was detected in numerous adrenocortical cells located in the zona glomerulosa, as well as in some cords of steroidogenic cells present in the zona fasciculata (Fig. 1). In the zona glomerulosa, 5-HT₄ receptor immunostaining was observed in clusters of steroidogenic cells which were either immunopositive or immunonegative for CYP11B1 (11 β -hydroxylase), suggesting that glucocorticoid producing cells present in the subcapsular region of the cortex express 5-HT₄ receptors. An intense 5-HT₄ receptor immunolabeling was detected at the periphery of CYP11B1-positive cells, indicating that the receptors are addressed to the plasma membrane in cortisol-producing cells.

3.2. cAMP and protein kinase A mediate the cortisol response to 5-HT

Application of 5-HT (10^{-6} M) to human adrenocortical fragments increased cAMP production by $72 \pm 8\%$ (Fig. 2A). The stimulatory effect of 5-HT on cAMP synthesis was blocked by the 5-HT₄ receptor antagonist GR113808 (10^{-6} M). As previously reported (Contesse et al., 2002), 5-HT (10^{-6} M) stimulated cortisol secretion by cultured adrenocortical cells (Fig. 2B). The cortisol response to 5-HT (10^{-6} M) was inhibited by the protein kinase A (PKA) inhibitor H-89 in a dose-dependent manner, but was not affected by the protein kinase C inhibitor chelerythrine (10^{-5} M; Fig. 2C). These data indicate that the 5-HT₄ receptor is positively coupled to the adenylyl cyclase/PKA pathway in human cortisol-producing adrenocortical cells.

3.3. Activation of 5-HT₄ receptors increases cytosolic Ca²⁺ concentration

In order to investigate the coupling of 5-HT₄ receptors to Ca²⁺ signal, intracellular calcium concentration ($[Ca^{2+}]_i$) was measured by microfluorimetry. Application of 5-HT (10^{-5} M) in the vicinity of cultured adrenocortical cells increased $[Ca^{2+}]_i$ from 65 ± 7 nM to 242 ± 27 nM ($n = 20$; $p < 0.0001$) (Fig. 3A). The $[Ca^{2+}]_i$ response to 5-HT developed slowly, peaked within 205 ± 38 s, and then declined gradually. 5-HT-induced $[Ca^{2+}]_i$ increase was mimicked by the 5-HT₄ receptor agonist zacopride (10^{-6} M; from 61 ± 11 nM to 369 ± 69 nM; $n = 9$; $p = 0.0004$) (Fig. 3B). Fig. 3C shows that 5-HT stimulated $[Ca^{2+}]_i$ and secretion of cortisol with similar potencies ($pEC_{50} = 7.14 \pm 0.01$ and 7.43 ± 0.20 , respectively).

3.4. Involvement of cAMP in the Ca²⁺ response to 5-HT

Administration of the cell-permeable cAMP analog dbcAMP (10⁻⁴M) provoked an increase in [Ca²⁺]_i from 56 ± 6 nM to 276 ± 62 nM (n = 6; *p* = 0.005; Fig. 3D). In addition, the phosphodiesterase inhibitor IBMX (10⁻⁴ M) enhanced the [Ca²⁺]_i response to 5-HT (10⁻⁵ M) up to 405 ± 61 nM (n = 8; *p* = 0.008; Fig. 3E). These results indicate that cAMP mediates the impact of 5-HT₄ receptors on Ca²⁺ signaling in human adrenocortical cells.

3.5. Contribution of extracellular Ca²⁺ to cortisol response to 5-HT

To evaluate the role of Ca²⁺ influx in the responses of adrenocortical cells to 5-HT, extracellular Ca²⁺ concentration was reduced by using the Ca²⁺ chelator EGTA. As shown by microfluorimetric studies, addition of EGTA during the [Ca²⁺]_i rise provoked a rapid and reversible decrease in [Ca²⁺]_i evoked by 5-HT or zacopride (Fig. 4A). Calcium dependence of the cortisol response to 5-HT was also investigated by reducing the extracellular Ca²⁺ concentration from 1.8 mM to 6 μM (Fig. 4B). Diminution of extracellular Ca²⁺ concentration decreased the amplitude of maximum response, indicating that efficacy of signal transduction was reduced (E_{max} = 243 ± 4% at 1 mM Ca²⁺ to 170 ± 5% at 0.01 mM Ca²⁺). By contrast, reduction in extracellular Ca²⁺ concentration did not affect half maximal effective concentration of 5-HT to stimulate cortisol secretion (potency; pEC₅₀ = 7.7 ± 1.7 at 1 mM Ca²⁺ and 7.8 ± 1.6 at 0.01 mM Ca²⁺), suggesting that affinity of 5-HT₄ receptor for the indolamine was not modified. In addition, perfusion experiments revealed that chelation of extracellular Ca²⁺ markedly attenuated the cortisol response of adrenocortical explants to zacopride (Fig. 4C).

3.6. Involvement of T-type Ca²⁺ current in cortisol response to 5-HT

Electrophysiological recordings were performed to study the effect of 5-HT on T-type Ca²⁺ current. Resting potential of cultured adrenocortical cells was measured between -70 mV and -60 mV. Cultured cells did not display spontaneous electrical activity (data not shown). As previously demonstrated (Barrett et al., 2016; Enyeart and Enyeart, 2015; Rossier, 2016; Scholl et al., 2015), cultured human adrenocortical cells emitted low-threshold transient inward Ca²⁺ currents, identified as T-type currents, in response to increasing depolarizing pulses higher than -60 mV (Fig. 5A). Application of 5-HT (10⁻⁶ M, 3 min) enhanced the amplitude but did not modify the activation threshold of T-type Ca²⁺ currents (Fig. 5B).

Since human adrenocortical cells are known to express different types of membrane Ca²⁺ channels (Enyeart and Enyeart, 2015; Felizola et al., 2014; Scholl et al., 2015, 2013), specific Ca²⁺ channel blockers have been used to identify the source of Ca²⁺ involved in the corticotropic effect of 5-HT. Incubation of cultured adrenocortical cells with the T-type channel blockers mibefradil (10⁻⁶ M) or NiCl₂ at low concentration (8 × 10⁻⁵ M) inhibited the cortisol response to 5-HT by reducing the maximum efficacy and/or potency (Fig. 5C). In addition, the L-type channel blocker nifedipine (10⁻⁵ M) reduced spontaneous and 5-HT-evoked cortisol production, whereas the N-type channel blocker ω-conotoxin (10⁻⁶ M) decreased the spontaneous secretion of glucocorticoid but did not significantly modify the magnitude of the stimulatory effect of 5-HT on cultured adrenocortical cells (Fig. 5D). The

P-type channel blocker sFTX-3.3 (10^{-6} M) had no effect on spontaneous or 5-HT-evoked cortisol secretion.

4. Discussion

Both *in vivo* and *in vitro* experiments have formerly shown that, in human, 5-HT stimulates corticosteroid production through 5-HT₄ receptors. The present study demonstrates that activation of 5-HT₄ receptors stimulates adenylyl cyclase activity and Ca²⁺ influx through T-type Ca²⁺ currents that provoke an increase in cortisol secretion.

RT-PCR analysis have previously demonstrated that human adrenals express *HTR4* mRNAs encoding the 5-HT₄ receptors (Cartier et al., 2005). However, the expression pattern of this receptor in adrenal cortex has not been clearly established. Indeed, *HTR4* mRNAs have been observed both in glomerulosa and fasciculata zonae by *in situ* hybridization (Cartier et al., 2005), whereas the protein has been mainly detected in zona glomerulosa by binding studies and immunohistochemistry (Kuc et al., 2005; Lefebvre et al., 2015). The present immunofluorescent study confirms that 5-HT₄ receptors are intensely expressed in the zona glomerulosa and in some cell cords in the zona fasciculata. This heterogeneous distribution of 5-HT₄ receptors in the cortex may explain the higher efficacy of 5-HT to activate mineralocorticoid secretion than glucocorticoid production (Lefebvre et al., 1993, 1992). Expression of 5-HT₄ receptor has also been reported in frog adrenocortical cells, whereas this receptor subtype is not present in rat adrenal (Contesse et al., 2000, 1996). In this rodent, the action of 5-HT on aldosterone secretion is mediated by 5-HT₇ receptors (Lenglet et al., 2002). Our previous studies have demonstrated that 5-HT₇ receptor is not expressed in normal human adrenocortical cells (Bram et al., 2016; Louiset et al., 2006), while abnormal expression of functional 5-HT₇ receptors has been detected in adrenal neoplasia from patients with primary hypercortisolism (Bram et al., 2016; Louiset et al., 2008, 2006). These data demonstrate that expression pattern of 5-HT receptor subtypes varies in a species-specific manner or between physiological and pathophysiological conditions.

Our double immunohistological study gives evidence for the presence of cells immunoreactive to both 11 β -hydroxylase and 5-HT₄ receptor in the zona glomerulosa, indicating that 5-HT released by mast cells present in the subcapsular region, may exert a direct control of glucocorticoid-producing cells through activation of 5-HT₄ receptors.

Because 5-HT₄ receptors have been reported to be positively coupled to the cAMP/PKA pathway in different tissues (Castro et al., 2005; Huang and Kandel, 2007; Weninger et al., 2014), we have first focused the study of their transduction mechanism in human adrenals on this pathway. The observations that, on one hand, 5-HT increased cAMP production and, on the other hand, the 5-HT₄ receptor antagonist GR113808 abolished 5-HT-induced cAMP formation, demonstrate that the 5-HT₄ receptors are also positively coupled to adenylyl cyclase in human adrenals. Coupling of 5-HT₄ receptors to the adenylyl cyclase/PKA pathway was confirmed by the inhibitory effect of the PKA inhibitor H-89 on the cortisol response to 5-HT. Thus, in human cortisol-producing cells, 5-HT₄ receptors activate the same second messenger system as MC2R (Gallo-Payet, 2016; Gallo-Payet and Payet, 2003).

It is well established that in heart, 5-HT₄ receptors exert prokinetic effects by activating L-type Ca²⁺ currents (Birkeland et al., 2007; Levy et al., 2008; Pau et al., 2005). Interestingly, 5-HT₄ receptors have been reported to increase aldosterone secretion by stimulating T-type Ca²⁺ currents in frog adrenocortical cells (Contesse et al., 1996). On the basis of these data, we have next investigated the involvement of Ca²⁺ in the response of human adrenocortical cell to 5-HT. We observed that 5-HT and the 5-HT₄ receptor agonist zacopride generated a slow and robust increase in [Ca²⁺]_i, as previously reported for ACTH in rat and bovine glomerulosa cells (Gallo-Payet et al., 1996; Tremblay et al., 1991). The fact that 5-HT-induced increase in [Ca²⁺]_i was mimicked by the cAMP analog dbcAMP and potentiated by the phosphodiesterase inhibitor IBMX indicate that cAMP, produced in response to activation of 5-HT₄ receptors, mediated the Ca²⁺ response.

In our adrenocortical cell cultures, *CYP11B1* mRNA level was almost 700 times higher than *CYP11B2* transcript level indicating that cortisol-producing cells is by far the most abundant cell population (personal data). We have thus compared the variation of [Ca²⁺]_i with glucocorticoid synthesis. 5-HT exerts its positive effects on [Ca²⁺]_i and cortisol secretion with similar efficacies and potencies, suggesting that these processes are closely coupled. Moreover, the Ca²⁺ and cortisol responses to 5-HT seem to be both due to Ca²⁺ influx into adrenocortical cells, since they were reduced by decrease in Ca²⁺ concentration obtained by addition of the Ca²⁺ chelator EGTA in the incubation medium. These data indicate that the Ca²⁺ signal relays the action of 5-HT on steroid synthesis. Consistently, Cherradi et al. have clearly demonstrated that increases in intracellular Ca²⁺ exert positive effects on actors of steroidogenesis, including the hormone-sensitive lipase and the steroidogenic acute regulatory protein Star (Cherradi et al., 2003, 1998).

Different types of membrane Ca²⁺ channels have been identified in human adrenal cortex by RT-PCR analysis and immunohistochemistry (Felizola et al., 2014; Scholl et al., 2015, 2013). In particular, expression of the L-, N- and T-type channels has been documented with a predominant localization in zona glomerulosa. In addition, L- and T-type Ca²⁺ currents have been recorded in steroidogenic cells from the glomerulosa and fasciculata zonae (Barrett et al., 2016; Enyeart and Enyeart, 2015, 2013; Payet et al., 1994; Rossier, 2016). Thus, we have hypothesized that 5-HT-induced Ca²⁺ influx in adrenocortical cells may be mediated by these channels. By using the patch-clamp technique, we found that 5-HT weakly enhanced the T-type Ca²⁺ currents. Nevertheless, inhibition of the cortisol response to 5-HT by the T-type channel blockers mibefradil and low dose of Ni²⁺ indicates that T-type channels are actually involved in 5-HT-induced steroidogenesis. In addition, it can be speculated that Ca²⁺ entry occurs, at least in part, *via* T-type Cav3.2 channels owing to the extreme sensitivity of these channels to Ni²⁺ (Enyeart and Enyeart, 2013). It seems thus that Ca²⁺ influx through T-type channels could provide a membrane depolarization sufficiently intense to activate L-type and N-type Ca²⁺ channels. Our observation that the L-type Ca²⁺ channel blocker nifedipine inhibited spontaneous and 5-HT-evoked cortisol secretions supports this hypothesis.

Globally, our results demonstrate that 5-HT₄ receptors are positively coupled to adenylyl cyclase. The activation of cAMP-dependent protein kinases causes Ca²⁺ influx through T-type Ca²⁺ channels that in turn stimulates cortisol production. The second messenger system

of 5-HT₄ receptors in human adrenocortical cells is thus similar to that of 5-HT₇ receptors expressed in rat glomerulosa cells (Lenglet et al., 2002).

Although the transduction pathway of 5-HT₄ receptors and MC2R are identical in human adrenocortical cells, it can be noticed that 5-HT is much less efficient than ACTH to activate cortisol secretion. This observation raises the question of the role of 5-HT₄ receptors in the control of glucocorticoid synthesis. We can speculate that activation of 5-HT₄ receptors may reinforce the action of ACTH in particular physiological conditions. The fact that 5-HT is produced by mast cells which are innervated by sympathetic fibers, suggests that the serotonergic system exerts a corticotropic effect during acute stress or inflammation.

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Abbreviations

5-HT	5-hydroxytryptamine, serotonin
5-HT₄	5-HT receptor type 4
ACTH	adrenocorticotrophic hormone
MC2R	melanocortin type 2 receptor
PKA	protein kinase A
APCC	aldosterone-producing cell clusters
PLC	phospholipase C
IP₃	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
([Ca²⁺]_i)	intracellular calcium concentration

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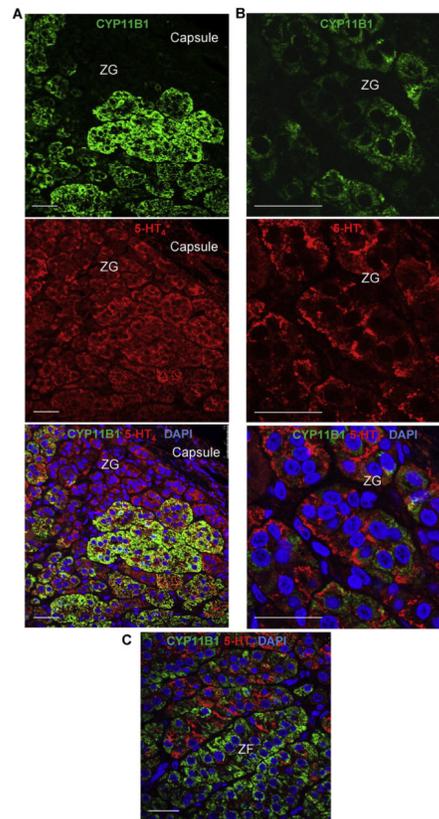


Fig. 1. Localization of 11 β -hydroxylase (CYP11B1) and 5-HT₄ receptors in human adrenal by immunohistofluorescence. (A, B) CYP11B1 (green; higher panels) and 5-HT₄ receptor (red; middle panels) immunoreactivities in adrenocortical cells in the zona glomerulosa (ZG) of the cortex at low (A) and high (B) magnifications. Merge (lower panels). (C) CYP11B1 and 5-HT₄ receptor immunoreactivities in the zona fasciculata (ZF). Nucleus stained with DAPI (blue). Scale bars, 50 μ m.

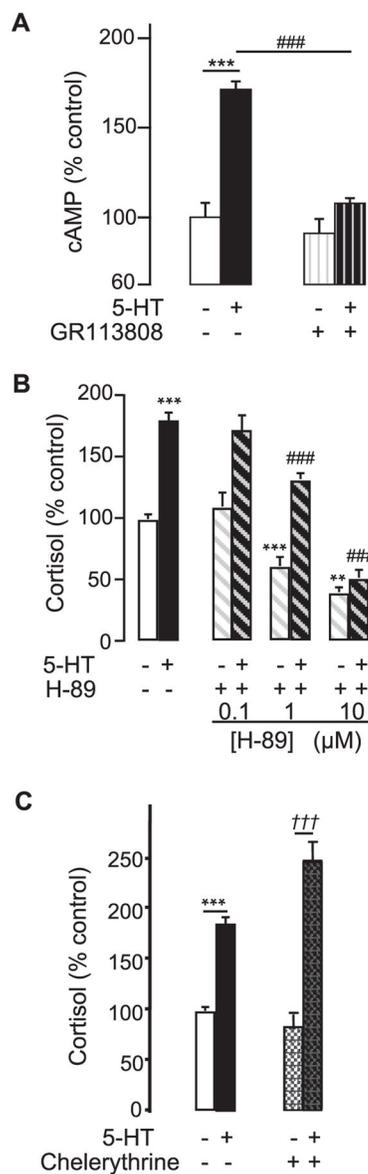


Fig. 2. Involvement of the cAMP/PKA pathway in the effect of 5-HT in human adrenocortical cells. (A) Effect of 5-HT (10^{-6} M) on cAMP production by human adrenal fragments incubated for 30 min in the absence or presence of the 5-HT₄ receptor antagonist GR113808 (10^{-6} M). (B, C) Cortisol production by cultured adrenocortical cells incubated for 24 h with or without 5-HT (10^{-6} M) in the absence or presence of increasing concentration of the PKA inhibitor H-89 (0.1, 1 and 10 μ M; B) and the PKC inhibitor chelerythrine (10^{-5} M; C). Data are expressed as mean \pm sem of at least three different experiments. **, $p < 0.01$; ***, $p < 0.001$ versus basal production in the absence of test substance. ##, $p < 0.01$; ###, $p < 0.001$ versus production in the presence of 5-HT. †††, $p < 0.001$ versus production in the presence of chelerythrine.

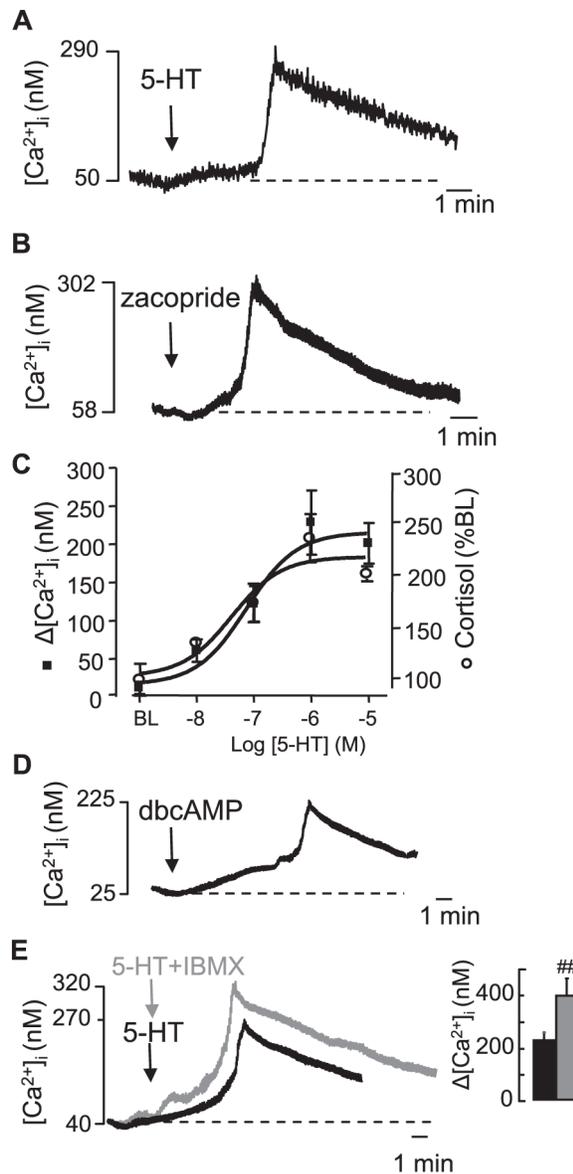


Fig. 3.

Effect of 5-HT on intracellular calcium concentration ($[Ca^{2+}]_i$) and cortisol secretion in cultured human adrenocortical cells. (A, B) Effect of 5-HT (10^{-5} M; A) and the 5-HT₄ receptor agonist zacopride (10^{-4} M; B) on $[Ca^{2+}]_i$. (C) Concentration-response curves showing the effects of 5-HT on $[Ca^{2+}]_i$ (■) and cortisol secretion (○). The data illustrate the mean \pm sem of maximum $[Ca^{2+}]_i$ measured from a series of 20 records similar to those shown in (A) and cortisol production induced by incubation of 5-HT for 24 h on 5 different cultures. (D, E) Effect of the cAMP analog dbcAMP (10^{-4} M; D) and 5-HT (10^{-5} M) in the absence or presence of the phosphodiesterase inhibitor IBMX (10^{-4} M; E *left*) on $[Ca^{2+}]_i$. *Right*, Peak of $[Ca^{2+}]_i$ measured in response to 5-HT alone (black) and in combination with IBMX (grey). Data represents mean \pm sem of 8 independent experiments; ##, $p < 0.01$. Arrows indicate the ejections for 30 s of test substances in the vicinity of studied cells.

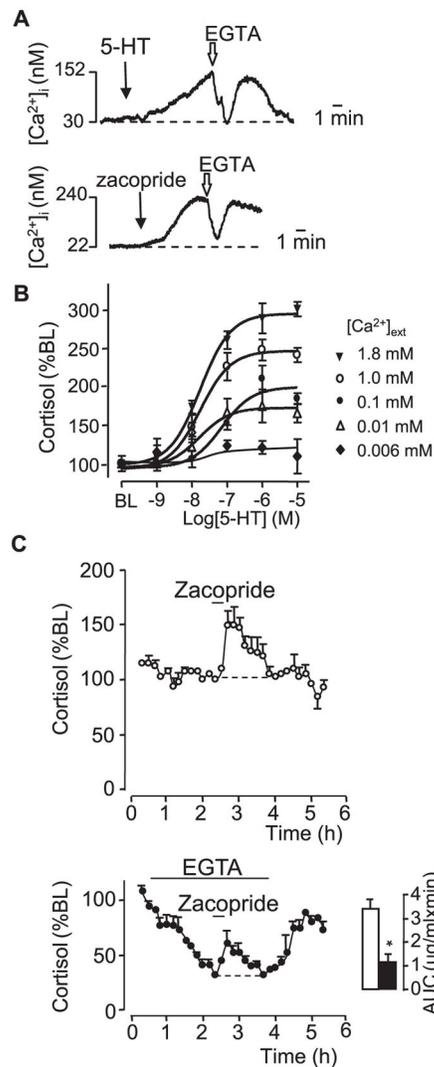


Fig. 4.

Effects of reduction of extracellular calcium concentration on $[Ca^{2+}]_i$ and cortisol responses to 5-HT in human adrenocortical cells. (A) Effect of the calcium chelator EGTA (10^{-2} M, *white arrows*) on the $[Ca^{2+}]_i$ increase induced by 5-HT (10^{-5} M; *higher panel*) and the 5-HT₄ receptor agonist zacopride (10^{-4} M; *lower panel*). Dashed lines represent basal levels of $[Ca^{2+}]_i$. (B) Concentration-response relationships of 5-HT on cortisol production in presence of different concentrations of extracellular calcium (\blacktriangledown , 1.8 mM; \circ , 1 mM; \bullet , 0.1 mM; \triangle , 0.01 mM; \blacklozenge , 0.006 mM Ca^{2+}). (C) Effect of zacopride (10^{-5} M, 20 min) on cortisol production by perfused adrenocortical explants in the absence (*upper panel*) or in presence of EGTA (10^{-2} M, 220 min; *lower panel, left*). The data represent the mean \pm sem of 3 independent experiments. The mean secretion rate in basal condition (100% basal level; BL) was 71.9 ± 10.8 pg/mg wet tissue per min. Dashed lines represent the mean cortisol production 5 min before administration of zacopride. Comparison of the area under the curve (AUC) for cortisol over time measured in the absence (white) or in presence of EGTA (black; *right panel*). *, $p < 0.05$.

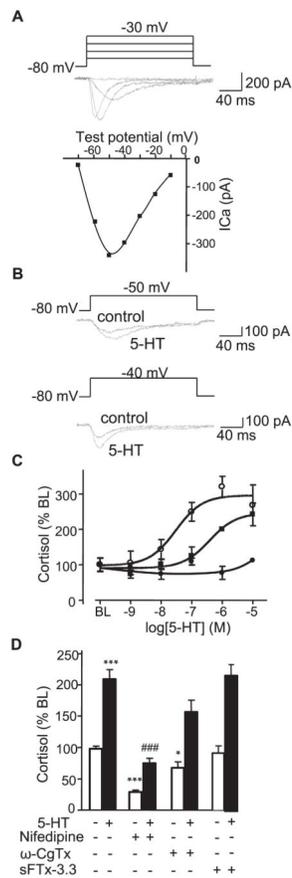


Fig. 5. Coupling of 5-HT receptors to T-type Ca^{2+} currents in human cultured adrenocortical cells. (A) Superimposed Ca^{2+} currents elicited by increasing depolarizing pulses (from -60 mV to -30 mV) from a holding potential of -80 mV (*upper panel*). Current-voltage relationships of the peak Ca^{2+} currents (*lower panel*). (B) Superimposed Ca^{2+} currents elicited by depolarizing pulses from -80 mV to -50 mV and -40 mV recorded in the absence (control) or presence of 5-HT (10^{-5} M). (C) Effects of graded concentrations of 5-HT on cortisol secretion by adrenocortical cells cultured in the absence or presence of the T-type Ca^{2+} channel blockers mibefradil (10^{-6} M; ■) and NiCl_2 (8×10^{-5} M; ●). (D) Effects of the L-type channel blocker nifedipine (10^{-5} M), N-type channel blocker ω -conotoxin (10^{-6} M) and P-type channel blocker sFTX-3.3 (10^{-6} M) on basal and 5-HT-induced cortisol production. BL, Basal level. *, $p < 0.05$; ***, $p < 0.001$ versus basal production in the absence of test substance. ###, $p < 0.001$ versus production in the presence of 5-HT.