EXPERIMENTAL STUDY

Expression profile of serotonin₄ (5-HT₄) receptors in adrenocortical aldosterone-producing adenomas

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Abstract

Objective: We aimed to investigate the expression profile of serotonin₄ (5-HT₄) receptors in adrenocortical aldosterone-producing adenoma (APA) tissues in comparison with normal adrenal cortex.

Design and methods: Total 5-HT₄ receptor mRNAs were quantified by real-time quantitative polymerase chain reaction (PCR) assay, and the mRNAs encoding the 5-HT₄ receptor isoforms were characterized by reverse transcription (RT)–PCR in seven normal adrenal cortices and 11 APA tissues. The distribution of 5-HT₄ receptor mRNAs was investigated by *in situ* hybridization in both normal adrenal and APA tissues, and the presence of 5-HT in APA tissues was studied by immunohistochemistry. **Results:** Real-time PCR analysis revealed that 5-HT₄ receptor mRNA expression was 4.7–47 times higher in APA tissues than in normal glands. *In situ* hybridization studies showed that 5-HT₄ receptor mRNAs were expressed in both zona glomerulosa and zona fasciculata/reticularis of the normal cortex and in groups of APA steroidogenic cells disseminated in the tumor tissues. Characterization of 5-HT₄ receptor splice variants by RT–PCR revealed different profiles of expression in APAs versus normal adrenals. Isoforms (a) and (b) were not expressed in any APA but were present in the majority of normal adrenocortical tissues. Conversely, isoform (d) was expressed in 5/11 APAs but only in 1/7 adrenals. Immunohistochemical studies revealed the presence of 5-HT-immunoreactivity in both mast cells and clusters of steroidogenic cells in APA tissues.

Conclusion: Our results show overexpression and different splicing of the 5-HT₄ receptor in APA tissues in comparison with normal adrenocortical tissue. They also demonstrate the presence of 5-HT in both mast cells and tumor steroidogenic cells, providing evidence for a possible autocrine/paracrine activation of aldosterone secretion within adenoma tissues.

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Introduction

Serotonin (5-HT) exerts its physiologic effects through activation of several subtypes of G protein-coupled receptors, including the serotonin₄ (5-HT₄) receptor (for a review, see Hoyer *et al.* (1)). Several studies have shown that, in man, the 5-HT₄ receptor primary transcript is alternatively spliced to produce eight receptor isoforms that differ in the length and structure of their C-terminal tail (a–g, n) and one variant which exhibits a 14-amino-acid insertion within the second extracellular loop (h) (2–5). Recently, a tenth isoform was cloned from the human pancreas and testis tissues and named 5-HT₄(i) (6). This isoform contains a 40-amino-acid insertion in the C-terminal tail of the previously known 5-HT₄(b) receptor (6). All isoforms are positively coupled to adenylyl cyclase and selectively activated by substituted benzamide derivatives such as metoclopramide, cisapride and zacopride (2–6).

We have previously shown that, in the human adrenal gland, 5-HT stimulates aldosterone and cortisol secretion through activation of 5-HT₄ receptors positively coupled to adenylyl cyclase and calcium influx (7–9). Reverse transcription (RT)–PCR studies have revealed that the human adrenal cortex expresses isoforms (a–d, f, h and n) (4, 10). We have also found that 5-HT can activate steroidogenesis in presumably autonomous adrenocortical lesions. An increased efficacy of 5-HT and/or 5-HT₄ receptor agonists to stimulate steroidogenesis was even observed in some cases of adrenocorticotrophic hormone (ACTH)-independent macronodular bilateral adrenal hyperplasia (AIMAH) and adrenocortical adenoma causing subclinical or overt Cushing's syndrome (10–12). In most cases, the hyperresponsiveness of AIMAH tissues to 5-HT₄ receptor agonists can be accounted for by an overexpression of 5-HT₄ receptors in comparison with normal adrenocortical tissue (10, 12), but the 5-HT₄ receptor variants expressed by AIMAH tissues are identical to those present in normal adrenocortical

tissue (10). Functional 5-HT₄ receptors are also expressed in aldosterone-producing adenomas (APAs). Indeed, oral administration of the 5-HT₄ receptor agonist cisapride to patients with APA causes an increase in plasma aldosterone levels (13). In addition, 5-HT and cisapride both stimulate aldosterone secretion from perfused APA explants (13). Finally, RT-PCR analysis has shown the presence of mRNA encoding the 5-HT₄ receptor in APA extracts (13). However, the amounts of 5-HT₄ receptor mRNA and the expression profile of 5-HT₄ receptor isoforms have never been determined in APA tissues.

In the normal human adrenal cortex, perivascular mast cells can release substantial amounts of 5-HT, which is likely to enhance corticosteroid secretion through paracrine mechanisms (7, 14). The presence of 5-HT has also been detected by immunohistochemistry in adrenocortical cortisol-producing lesions, including AIMAHs and adenomas (11, 12). In these tissues, 5-HT-like immunoreactivity is localized in a subpopulation of steroidogenic cells. APAs have been shown to contain numerous mast cells (15), suggesting that 5-HT may be responsible for paracrine activation of aldosterone secretion by APA cells. However, the occurrence of 5-HT in APA tissues has never been investigated.

The aim of the present study was to investigate quantitatively and qualitatively the expression of the 5-HT₄ receptor in both APA tissues removed from 11 patients and normal adrenal cortex. Quantitative RT-PCR was performed in order to compare the levels of expression of the 5-HT₄ receptor in APA tissues with those of normal adrenal cortex. The distribution of the 5-HT₄ receptor mRNA in both normal and aldosteronoma tissues was investigated by *in situ* hybridization, and the 5-HT₄ receptor variants expressed by the tissues were characterized by RT-PCR and subsequently subcloned and sequenced. Finally, the possible occurrence of 5-HT within the adenoma tissues was investigated by immunohistochemistry.

Materials and methods

Tissues, RNA extraction and real-time RT-PCR

APA tissues were provided by a French endocrinologic network for collection of adrenocortical tumors

(Réseau COMETE, PHRC AOM 95 201) and stored at -80°C until RNA extraction. Normal adrenal glands were obtained from seven patients undergoing expanded nephrectomy for kidney cancer. The protocol for collection of the tissues and the experimental procedures were approved by the regional and/or institutional ethics committees, and informed consent was obtained from all subjects. Total RNA from the 11 APAs and seven normal adrenal glands was extracted by the acid guanidium-thiocyanate-phenol-chloroform procedure with Tri-Reagent (Sigma). The concentration of total RNA was determined by measuring the optical density at 260 nm. Real-time RT-PCR analysis was carried out as described by Fink *et al.* (16) in order to quantify 5-HT₄ receptor mRNA in both aldosteronoma and normal adrenal cortex. The primers and fluorogenic TaqMan probe used for these experiments hybridized to all 5-HT₄ receptor splice variants (5-HT₄pan) (Table 1). Briefly, 1 μg total RNA from each tissue was converted to single-stranded cDNA using SuperScript II (Life Technologies, Eragny, France) with oligo (dT)12-18 primer (0.5 $\mu\text{g}/\text{ml}$), and the cDNA was diluted and aliquoted into microtiter plates. For each 25 μl TaqMan reaction, 5 μl cDNA were mixed with 1 μl water, 12.5 μl TaqMan Universal PCR Master Mix $\times 2$ (Applied Biosystems, Courtaboeuf, France), 2 μl sense primer (2 μM), 2 μl antisense primer (2 μM) and 2.5 μl TaqMan probe (2 μM). PCR parameters were 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. Parallel assays using the same cDNA pools were carried out with primers and probe to the housekeeping gene porphobilinogen deaminase (PBGD) (Table 1). Quantitative RT-PCR was performed with the ABI Prism 7700 sequence detector system (Applied Biosystems) and analyzed with relative expression to PBGD, as previously described (16). Briefly, the level of expression in each sample was normalized by dividing copies/ng total RNA of 5-HT₄ receptor gene by copies/ng total RNA of the housekeeping gene PBGD, and expressed as a percentage. This mode of calculation allows correction for both RNA quality and quantity. In each group of tissues, that is, normal and APAs, data are presented as mean $\pm$ S.E.M.

Table 1 Real-time PCR analysis of 5-HT₄ receptor mRNA.

cDNA	Accession No. in GenBank	Primer	Sequence (5'-3')
5-HT ₄ pan	Y 10437	s	AACGGCATCGATTTTTCACC
		as	GTTCCATAGACCAAAGGCTGGC
		p	TGCTGCATTCTCTGGATAGGTATTACGCC
PBG-D	X 04217	s	CTGCAACGGCGGAAGAA
		as	AGCTGGCTCTTGCGGGTAC
		p	CCCAAAGATGAGAGTGATTTCGCGTGG

Oligonucleotide sequences for sense (s) and antisense (as) primers and TaqMan probes (p) are shown.

In situ hybridization

Sense and antisense probes were prepared by *in vitro* transcription of a 1081 bp fragment of the human 5-HT₄ receptor cDNA encompassing exons I to V (position 3–1083; GenBank accession no. AJ 278982; this sequence is common to all 5-HT₄ receptor isoforms) and subcloned in the pGEM-T vector (Promega). The probes were labeled with ³⁵S-UTP (Amersham), using the Riboprobe Combination System (Promega). Frozen tissue sections (12-µm-thick) were initially fixed in cold 4% paraformaldehyde for 10 min, delipidized in chloroform and dehydrated in graded alcohols. Then, sections were acetylated, treated with Triton X-100 (0.2%), rinsed in 2 × standard saline citrate (SSC) and prehybridized at 55 °C for 1 h in the following buffer: 50% formamide, 0.6 M NaCl, 10 mM Tris–HCl (pH 7.5), 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.2% BSA, 1 mM EDTA (pH 8), 550 µg/ml denatured salmon sperm DNA and 50 µg/ml yeast tRNA. Hybridization was performed overnight, at 55 °C, in the same buffer (except for salmon sperm DNA, the concentration of which was lowered to 60 µg/ml) supplemented with 10 mM dithiothreitol, 10% dextran sulfate and heat-denatured riboprobes (1.5 × 10⁷ c.p.m./ml). Posthybridization washes were performed in 2 × SSC at 55 °C for 30 min, followed by treatment with RNase A (50 µg/ml) at 37 °C for 1 h, and by five final stringent washes in 0.1 × SSC at 60 °C. After dehydration in graded alcohols containing ammonium acetate, the sections were dipped into Kodak NTB2 emulsion, exposed for 6 weeks and counterstained with hematoxylin. Sections were observed with an Eclipse E600 microscope equipped with a CCD DXC950 camera (Nikon, Les Ulis, France).

Characterization of 5-HT₄ receptor isoforms by RT-PCR

Total RNA was extracted and reverse transcribed as described above. Amplification of the cDNAs encoding the different 5-HT₄ receptor C-terminal

splice variants was performed by PCR using primer S1 (Table 2), which hybridizes to all 5-HT₄ receptor messengers, and splice variant-specific reverse primers (Table 2 and Fig. 1). The 5-HT₄(h) variant was amplified with the forward primer Fwh, which is specific for cDNAs containing the 5-HT₄(h) exon, and the reverse primer Revh, which hybridizes to all 5-HT₄ receptor messengers. All PCR-based procedures were performed in a final volume of 50 µl containing 10% of RT mixture, 3 U DNA Taq polymerase (Life Technologies), DNA polymerase buffer (Life Technologies), 1.5 mM MgCl₂, 0.4 mM dNTP and 20 pmol of each primer. The PCR reactions were performed for 40 cycles (94 °C, 40 s; 50 °C, 60 s; 72 °C, 90 s). The PCR products were analyzed in 1.5% agarose gels, blotted on a nylon membrane and hybridized with the [³²P]ATP-labeled oligonucleotide S2. In addition, PCR products were subcloned into pGEM-T (Promega) and sequenced, using the Thermosequenase kit (Amersham), on a Li-Cor 4200L DNA sequencer (ScienceTec, Les Ulis, France) with fluorescent T7 and T3 primers (MWG-Biotech, Courtaboeuf, France).

Immunohistochemistry

Deparaffinized sections from APA tissues were incubated overnight at 4 °C in a humidified atmosphere with polyclonal rabbit antibodies directed against 5-HT (1:400; Sigma). The sections were then incubated with a streptavidin-biotin-peroxidase complex (Dako, Carpinteria, CA, USA), and the enzymatic activity was revealed with diaminobenzidine. The specificity of the immunoreaction was controlled by using 5-HT-antisera preabsorbed for 2 h at 20 °C with 10^{−6} M 5-HT. The tissue sections were counterstained for 1 min with hematoxylin, mounted in Eukitt (Kindler, Freiburg, Germany), cover-slipped, and examined on an Eclipse E-600 microscope equipped with a CCD DXC950 camera.

Table 2 Oligonucleotide sequences of sense and antisense primers used for RT-PCR analysis of 5-HT₄ receptor isoform mRNAs.

Primer	Accession No. in GenBank	Sequence (5'–3')
Fwh	AJ243213	GAAAGGAGTCTAAACCAAGGCCTGG
Revh	AJ243213	CATGTGTGGATCCATTAATGGTTGTGG
Fw1	Y10437	AATTGATTTGATAGAAAAGAGGAAG
Reva	Y08756	GTATGGGCAGTTTCTCGAGTTCCTGATGATG
Revb	Y12505	GAAGTTGCTGGCGGGTGACACTGACTCTC
Reve	Y12506	GGCATTAGGATGGTTTGGTCAATC
Revc	Y12507	CATCCAATGAATTTATTTGATAACTTCAG
Reve	AJ011371	CAGACGGGAACAGGTCTATTGCAGAAG
Revf	AJ243213	TTAGACGGGAACAGGTCTTAGTACATG
Revg	AJ243213	CAGAAGAGCAGGAGGAAGCTGGAGACAG
Revn	AJ278982	ACTGGAGCATTACCCCTTCTGAG
Revi	AJ131726	AAGACAGGCTTCCTTGCAGTCAAACATC
S2	Y10437	CCCTGGGCAGGTGTGGACTGC

Figure 1 Schematic representation of the human 5-HT₄ receptor gene showing the position of the primers used to amplify the 5-HT₄ receptor transcripts.

Results

Quantitative expression of 5-HT₄ receptors in normal adrenal glands and hyperplasias

The relative amounts of 5-HT₄ receptor mRNAs in normal adrenal cortex and aldosteronoma tissues were determined by real-time RT-PCR. When expressed as arbitrary units normalized to PBGD, 5-HT₄ receptor levels ranged from 0.48% to 3.72% (mean $2.13 \pm 0.46\%$) in normal adrenal glands vs 9.9–99.3% (mean $46.9 \pm 9.4\%$) in APAs (Fig. 2).

Distribution of 5-HT₄ receptor mRNA in normal adrenal cortex and APA tissues

In situ hybridization histochemistry showed that, in the normal adrenal cortex, 5-HT₄ receptor mRNA is primarily expressed in the zona glomerulosa (Fig. 3A and B). A weaker hybridization signal was also visualized in some cords of zona fasciculata/reticularis cells (Fig. 3A and B). Incubation of normal adrenal tissue sections with the sense riboprobe did not produce any staining in the zona glomerulosa nor in the zona

fasciculata/reticularis. In contrast, labeling was observed in the capsule and was thus considered non-specific (Fig. 3C). Incubation of APAs with the antisense probe produced a more intense and diffuse labeling than in the adrenal cortex (Fig. 4A and B). At a higher magnification, the hybridization signal was seen in groups of cells disseminated in the tissue and frequently arranged in acini (Fig. 4C). No labeling was observed when adjacent sections were incubated with the sense riboprobe (Fig. 4D).

Characterization of 5-HT₄ receptor isoforms by RT-PCR

RT-PCR amplification was applied to characterize 5-HT₄ receptor isoforms in both normal adrenal glands and APAs (Table 3). Isoforms (a) and (b), which were expressed in most normal adrenal extracts, were not detected in any of the APAs studied. Isoform (f), which was found in all normal adrenal cortices, occurred in only 5/11 APAs. In contrast, isoform (d) was more frequently expressed in APA tissues than in the normal cortex (5/11 vs 1/7 respectively). The profiles of expression of 5-HT₄ receptor isoforms (c), (e), (g), (h) and (n) were globally similar in APAs and normal cortex. Indeed, isoform (c) was expressed in 10/11 APAs and all normal cortices, and isoform (n) was found in all extracts; in contrast, isoforms (e), (g) and (h) were not detected in any of the tissues studied. We have also looked for the expression of isoform (i), which was identified while this study was in progress (6), in three normal adrenal cortices and the series of APAs. Isoform (i) mRNA was detected in one adrenal gland but did not occur in any APA (data not shown).

Figure 2 Expression levels of 5-HT₄ receptor mRNAs in seven normal human adrenal cortices and 11 APA tissues. TaqMan RT-PCR analysis was carried out with primers and a fluorogenic probe that recognized all 5-HT₄ splice variants (5-HT₄pan; see Table 3 for oligonucleotide sequences). 5-HT₄ receptor mRNA expression is in arbitrary units normalized to PBGD. The shaded area corresponds to the range of variation of expression levels observed in normal tissues.

Immunohistochemical detection of 5-HT in APA tissues

Incubation of APA slices with 5-HT antibodies revealed the presence of immunopositive cells disseminated in the whole tissue (Fig. 5A). The immunoreactive material was detected in isolated mast cells (Fig. 5B), as previously observed in the normal adrenal cortex (7). 5-HT-like immunoreactivity was also detected in a subpopulation of steroidogenic cells presenting as spongiocytic or

Figure 3 Localization of 5-HT₄ receptor gene expression by *in situ* hybridization in the normal adrenal cortex. (A) Microphotograph under epipolarization showing a positive hybridization signal in the capsule (c) and the zona glomerulosa (g; arrows), and a more sparse signal in some cords of zona fasciculata/reticularis cells (r/f; arrowheads). (B) Bright-field microphotographs of the same area of the adrenal cortex. (C) No labeling was observed in the zona glomerulosa and zona fasciculata/reticularis after incubation with the 5-HT₄ receptor sense riboprobe, while staining was still noticed in the capsule (arrowhead). Scale bars: 50 μ m.

compact cells (Fig. 5C). Preincubation of the 5-HT anti-serum with 5-HT (10^{-6} M) totally abolished immunostaining (data not shown).

Discussion

We have previously observed that APA tissues express functional 5-HT₄ receptors in very much the same way as the normal adrenal gland (13). We now show that, like the ACTH receptor (17), the 5-HT₄ receptor is markedly overexpressed in APAs in comparison with the normal adrenocortical tissue. Interestingly, the mean relative amount of 5-HT₄ receptor mRNA in APAs, that is, 46.9%, in arbitrary units normalized to PBGD, is also higher than that formerly reported in six 5-HT₄ receptor agonist-responsive AIMAHs (13.1%) and two unilateral adenomas (3.78% and 13.6% respectively) causing Cushing's syndrome (10, 12). This latter observation suggests that high expression of 5-HT₄ receptors, like high expression of aldosterone synthase (18), may be a characteristic of aldosterone-secreting adrenocortical tissues. We then performed *in situ* hybridization studies in order to investigate the distribution of 5-HT₄ receptor mRNAs in both normal adrenal gland and APAs. The results indicate that, in the adrenal cortex, 5-HT₄ receptor mRNAs are intensely expressed in the zona glomerulosa and in only scattered cords of zona fasciculata/reticularis cells. Conversely, we observed that 5-HT₄ receptor mRNAs are strongly expressed in a majority of APA cells. As the zona glomerulosa represents only a minor part of the adrenal cortex, this observation probably explains overexpression of total 5-HT₄ receptor mRNAs in APAs as compared with the normal adrenocortical tissue. The predominant expression of 5-HT₄ receptor mRNAs in the zona glomerulosa is also consistent with previous studies showing that 5-HT₄ receptor agonists are much more potent in stimulating *in vitro* aldosterone than cortisol secretion from normal adrenocortical explants (19). Collectively, the results of real-time PCR and *in situ* hybridization studies suggest that high expression of 5-HT₄ receptors by normal and tumor adrenocortical cells is a feature of glomerulal differentiation.

Previous studies have shown that, in COS-7 cells transfected with 5-HT₄ receptors, both basal and 5-HT₄ receptor agonist-stimulated cAMP productions increase as a function of receptor density (20). It would thus be expected that overexpression of 5-HT₄ receptor mRNA by APA tissues would induce increased responsiveness of these tumors to 5-HT₄ receptor agonists. In fact, we have previously observed that, in patients with APA, the amplitude of the plasma aldosterone response to the 5-HT₄ receptor agonist cisapride (that is, +133% of basal level, a value calculated from previously published data (13)) is lower than that observed in healthy subjects (+232% of basal level, a value calculated from previously published data (21)). The apparent discrepancy between the 5-HT₄ receptor expression data and the results of clinical studies may be ascribed to the fact that only part of the 5-HT₄ receptor mRNAs is translated into functional proteins in APA tissues. It could also

Figure 4 Localization of 5-HT₄ receptor gene expression by *in situ* hybridization in APA tissues. (A) Representative dark-field microphotograph showing intense staining of the tissue. (B) Bright-field microphotograph of the same area of the APA tissue. (C) A strong hybridization signal was observed in clusters of cells frequently arranged in acini (arrow). (D) No labeling was observed in the same region of the tissue after incubation with the 5-HT₄ receptor sense riboprobe. Scale bars: A, B and D, 50 μ m; C, 25 μ m.

be attributable to disparities in the expression profiles of 5-HT₄ receptor splice variants, which may result in functional variability in 5-HT₄ receptor-mediated tissue responses. Indeed, it is now well established that the diverse 5-HT₄ receptor isoforms may activate different transduction pathways in addition to adenylyl cyclase (for a review, see Bockaert *et al.* (22)). 5-HT₄ receptor isoforms may also form homo- and/or heterodimers that may possess distinct pharmacologic

and/or biochemical characteristics, as already shown for other G-protein-coupled receptors (23–25). We have thus characterized the 5-HT₄ receptor isoforms expressed by APA tissues and normal adrenal cortex. APAs were found to exhibit an expression profile of 5-HT₄ receptor splice variants different from that of the normal adrenal cortex. In particular, APAs do not express isoforms (a) and (b) which are frequently present in normal adrenocortical tissues. Beside their ability to

Table 3 RT-PCR analysis of 5-HT₄ receptor isoform (a–h, n) mRNAs in the 11 APAs (A1–A11) and seven normal adrenals (N1–N7).

	a	b	c	d	e	f	g	h	n
A1	–	–	+	+	–	+	–	–	+
A2	–	–	+	–	–	+	–	–	+
A3	–	–	+	+	–	+	–	–	+
A4	–	–	+	–	–	+	–	–	+
A5	–	–	+	+	–	–	–	–	+
A6	–	–	–	–	–	–	–	–	+
A7	–	–	+	+	–	–	–	–	+
A8	–	–	+	–	–	–	–	–	+
A9	–	–	+	+	–	+	–	–	+
A10	–	–	+	–	–	–	–	–	+
A11	–	–	+	–	–	–	–	–	+
N1	+	+	+	–	–	+	–	–	+
N2	+	+	+	+	–	+	–	–	+
N3	+	–	+	–	–	+	–	–	+
N4	–	–	+	–	–	+	–	–	+
N5	+	+	+	–	–	+	–	–	+
N6	+	+	+	–	–	+	–	–	+
N7	+	+	+	–	–	+	–	–	+

Figure 5 Immunohistochemical localization of 5-HT in APA tissues. (A) Low-magnification microphotograph showing groups of 5-HT-immunoreactive cells disseminated in APA tissue. (B) Close-up view of an isolated immunopositive mast cell (arrow). (C) Photomicrograph showing 5-HT-like immunoreactivity in large, lipid-loaded spongiocytic cells (arrows) and compact steroidogenic cells (arrow-head). Scale bars: 25 µm

activate the Gα_s protein, isoforms (a) and (b) are known to establish additional coupling mechanisms, probably leading to a more efficient transduction of the message. For instance, isoform (a) can also activate heterotrimeric Gα₁₃ protein, while isoform (b) can stimulate the MAP-kinase pathway, which is involved in calcium-induced aldosterone secretion (26–28). The loss of isoforms (a) and (b) may therefore reduce the efficacy of 5-HT₄ receptor agonists to stimulate aldosterone secretion in patients with APA. Similarly, the fact that isoform (d) is more frequently expressed in APAs than in normal tissues may account for the decreased steroidogenic response to cisapride, as this variant is more rapidly desensitized by 5-HT₄ receptor agonists than the other isoforms (29). Conversely, it is difficult to evaluate the functional consequences of the loss of isoform (i) and the less frequent isoform (f) expression in APAs, as compared with the normal cortex, since the transduction mechanisms associated with these variants have not yet been fully elucidated. In summary, the characterization of the expression profile of 5-HT₄ receptor isoforms in all the tissues studied has revealed that, unlike normal adrenals, APA cells preferentially express splice variants which are potentially less effective to induce the steroidogenic response. This observation may explain why the amplitude of cisapride-induced aldosterone secretion is higher in healthy volunteers than in APA patients despite overexpression of total 5-HT₄ receptor mRNA in the tumor tissues. The unusual splicing of the 5-HT₄ receptor pre-mRNA in APA cells may be considered a cell-adaptive mechanism aimed at limiting the stimulatory effect of 5-HT and subsequently the degree of hyperaldosteronism.

Biochemical and immunohistochemical studies have demonstrated that, in the normal adrenal cortex, 5-HT is exclusively stored and released by perivascular mast cells (7, 14). In cortisol-producing adrenocortical lesions, including bilateral macronodular hyperplasias and adenomas, 5-HT-like immunoreactivity is found in the cytoplasm of a subpopulation of steroidogenic cells

(11, 12). It has also been shown that APAs contain numerous mast cells (15). The present study now indicates that, in APAs, 5-HT is located in both mast cells and groups of steroidogenic cells, suggesting that 5-HT concentration in APA tissues is probably higher than in normal adrenal cortex. In addition, the detection of 5-HT in APA spongiocytic cells provides further evidence of the presence of hybrid cells, that is, those exhibiting both steroidogenic and neuroendocrine cell characteristics, in adrenocortical lesions (11, 12, 30, 31). These observations indicate that 5-HT may exert a tonic stimulatory effect on aldosterone secretion through an autocrine/paracrine mode of regulation and thus may play a significant role in the pathophysiology of APAs. In particular, 5-HT, together with other adenylyl cyclase-activating regulatory factors such as ACTH and luteinizing hormone, may participate in the previously shown overexpression of aldosterone synthase in APA tissues (18) through stimulation of the transcription factors Nurr1 and Nurr77 (17, 32–34).

In conclusion, the present study has shown that aldosterone-producing adenomas overexpress the 5-HT₄ receptor as compared with the normal adrenal cortex. Our results also demonstrate the existence of different splicing of the 5-HT₄ receptor transcript in APA. Finally, the data show for the first time that APAs produce 5-HT on their own, providing evidence of autocrine/paracrine regulation of aldosterone secretion within the tumor tissues. Collectively, these observations suggest that selective 5-HT₄ receptor antagonists may represent a new approach in the treatment of primary hyperaldosteronism.

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