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Overexpression of Serotonin$_4$ Receptors in Cisapride-Responsive Adrenocorticotropin-Independent Bilateral Macronodular Adrenal Hyperplasia Causing Cushing's Syndrome

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The serotonin$_4$ (5-HT$_4$) receptor agonists cisapride and/or metoclopramide have been shown to stimulate cortisol secretion in some patients with ACTH-independent bilateral macronodular adrenal hyperplasias (AIMAH) causing Cushing's syndrome. In the present study, we have investigated quantitatively and qualitatively the expression of the 5-HT$_4$ receptor in both normal adrenal cortex and tissues removed from six patients (P1–P6) with cisapride-responsive AIMAH and Cushing's syndrome. Real-time quantitative PCR assay revealed that the 5-HT$_4$ receptor was overexpressed in four of the six hyperplasias studied when compared with normal adrenal cortex. In these tissues, 5-HT$_4$ receptor mRNA expression was 3 to 16 times higher than in normal glands, likely explaining the abnormal *in vivo* cortisol response to cisapride. Characterization of 5-HT$_4$ receptor splice variants by RT-PCR in both hyperplastic and normal adrenals showed that the variants present in the two hyperplasias that did not overexpress the 5-HT$_4$ receptor, *i.e.* P2 and P5, could also be detected in the normal adrenal tissue. In addition, sequencing of the full-length cDNAs encoding 5-HT$_4$ receptors in hyperplasias P2 and P5 did not reveal any mutation. Taken together, our results show an overexpression of the 5-HT$_4$ receptor in cisapride-responsive AIMAH. However, in two cases, the level of expression of the receptor in the hyperplastic adrenal cortex was similar to that of normal adrenal gland. The enhanced sensitivity of these two tissues to 5-HT$_4$ receptor agonists was not due to ectopic expression of 5-HT$_4$ receptor isoforms or to the occurrence of somatic gain-of-function mutation of the receptor. (*J Clin Endocrinol Metab* 88: 248–254, 2003)

MOST PHYSIOLOGICAL EFFECTS of serotonin (5-HT) are mediated by a wide variety of 7-transmembrane domain G protein-coupled receptors, including the 5-HT$_4$ receptor (for review, see Ref. 1). Alternative splicing of the 5-HT$_4$ receptor transcript has the potential to generate eight receptor isoforms (a–g, and n) that differ in the length and structure of their C-terminal tail (2–4). The cloning of the 5-HT$_4$ receptor gene allowed the characterization of a ninth variant (isoform h) resulting from a 14-amino acid insertion within the second extracellular loop (5). All isoforms are positively coupled to adenylyl cyclase and activated by benzamide derivatives, such as metoclopramide, zacopride, and cisapride (1, 4, 5). It is now well demonstrated that 5-HT stimulates aldosterone and cortisol secretion from the human adrenal cortex through a paracrine mechanism involving 5-HT$_4$ receptors positively coupled to adenylyl cyclase and calcium influx (6–8). Interestingly, 5-HT$_4$ receptor agonists appear to be much more potent in stimulating *in vitro* aldosterone than cortisol secretion (9). In agreement with this latter observation, oral administration of zacopride and/or cisapride to healthy volunteers induces a significant increase in plasma aldosterone levels but does not influence plasma cortisol concentrations (9, 10).

Several observations indicate that cortisol production is controlled by aberrant membrane receptors in both adrenal tumors and ACTH-independent bilateral macronodular adrenal hyperplasias (AIMAH) causing Cushing's syndrome (11). Abnormal responses of cortisol secretion to administration of ligands for eutopic receptors have also been described in patients with primary adrenal hypercortisolism (11). In particular, cisapride and metoclopramide have been shown to stimulate cortisol secretion in patients with AIMAH and subclinical or patent Cushing's syndrome, suggesting an overexpression of 5-HT$_4$ receptors in this type of lesion (11, 12). However, the mechanisms involved in the

Abbreviations: AIMAH, ACTH-independent bilateral macronodular adrenal hyperplasias; CT, computerized tomography; 5-HT, serotonin; LVP, lysine vasopressin; nts, nucleotides; PBGD, porphobilinogen deaminase.

hyper-responsiveness of the adrenal hyperplastic tissue to 5-HT_4 receptor agonists remain currently unknown.

The aim of the present study was to investigate quantitatively and qualitatively the expression of the 5-HT_4 receptor in both normal adrenal cortex and tissues removed from six patients with cisapride-responsive AIMAH causing subclinical or patent Cushing's syndrome. Quantitative PCR was performed to compare the levels of expression of the 5-HT_4 receptor in hyperplastic adrenal tissues with those of normal adrenal cortices. In addition, the 5-HT_4 receptor variants expressed by the tissues were identified by use of RT-PCR and subsequently subcloned and sequenced.

Patients and Methods

Patients

Six patients with AIMAH causing subclinical or overt Cushing's syndrome were studied. Patient 1, a 73-yr-old woman, was referred for bilateral adrenal hyperplasia discovered incidentally by abdominal ultrasound. She had experienced hypertension for 8 yr. Clinical examination showed typical signs of hypercortisolism including facial plethora, epidermal atrophy, and proximal myopathy. Abdominal magnetic resonance imaging revealed a nodular hyperplasia of the two adrenal glands reaching a diameter of 3.5 cm on the right and 2.0 cm on the left. The clinical presentation of patient 2, a 63-yr-old woman, has been previously described (13). Briefly, she presented with a 12-month history of hypertension, proximal-muscle weakness, decrease in memory, and weight gain of 19 kg during the last 9 yr. She had four full-term pregnancies, at which time she had a Cushingoid distribution of fat and weight gain of 18–22 kg. Postpartum, her weight had rapidly returned to baseline. She had undergone hysterectomy and bilateral oophorectomy at 61 yr of age because of uterine prolapse. At examination, she had central obesity, mild facial plethora and hirsutism, supraclavicular fat pads, and proximal muscle weakness. An abdominal computerized tomography (CT) scan showed bilateral macronodular adrenal hyperplasia with nodules measuring up to 4 × 3.5 cm on the right and 2.5 × 4 cm on the left. Patient 3, a 50-yr-old woman, was admitted for bilateral adrenal tumors incidentally discovered during abdominal CT scan performed for lumbar pain. She had no history of hypertension. Her menses had stopped 6 months previously. Clinical examination revealed no sign of hypercortisolism. On abdominal CT scan, the adrenal nodules reached 5 × 3 cm on the right and 3 × 2 cm on the left. An iodocholesterol scan performed without dexamethasone suppression showed bilateral adrenal uptake of the tracer predominant on the right gland. Patient 4, a 46-yr-old man, presented with a history of weight gain, muscle weakness, and hypertension. Abdominal CT scan revealed enlarged adrenal glands with nodules measuring up to 2.5 cm on the right and 3.5 cm on the left. Patient 5 was a 34-yr-old woman referred for central obesity, hypertension, and diabetes mellitus. On abdominal CT scan, adrenal glands were hyperplastic with nodules measuring up to 2 cm on the left and 5 cm on the right. Patient 6, a 74-yr-old man, was referred for symptoms suggestive of hypercortisolism comprising facial plethora and erythrosis, easy bruisability, and hypertension. Abdominal CT scan showed multiple adrenal nodules reaching 5 cm on the left and 3 cm on the right.

The results of the initial endocrine investigations for the six patients are summarized in Table 1. Potentially aberrant membrane hormone receptors were systematically searched for, after informed consent of the patients, by using a clinical protocol previously described (14). The study was approved by the regional and/or institutional ethics committees. Briefly, plasma cortisol levels were measured in response to a posture test, a standard mixed meal, administration of 250 μg $ACTH_{1-24}$ iv used as a reference test, 100 μg GnRH iv, 100 μg TRH iv, 1 mg glucagon iv,

TABLE 1. Clinical characteristics and initial endocrine evaluation in the six patients with cisapride-responsive ACTH-independent bilateral macronodular adrenal hyperplasia

	P1	P2	P3	P4	P5	P6
Sex	Female	Female	Female	Male	Female	Male
Age (yr)	73	63	50	46	34	74
Symptoms of hypercortisolism	Hypertension Facial plethora Proximal amyotrophy	Hypertension Facial plethora Proximal amyotrophy	None	Hypertension Central obesity Proximal amyotrophy	Hypertension Central obesity	Hypertension Facial plethora Easy bruising
Urinary free cortisol (nmol/d)	145 (N: 55–248)	770 (N: 55–248)	135 (N: 54–190)	826 (N: 55–220)	1172 (N: 55–248)	356 (N: 55–276)
Plasma cortisol (nmol/liter)						
0800 h	373 (N: 200–700)	784 (N: 175–700)	324 (N: 273–436)	806 (N: 250–850)	510 (N: 276–552)	463 (N: 120–618)
Between 1600 h and 2000 h	264	508	319	759	466	397
Plasma ACTH at 0800 h (pmol/liter; N: 2–12)	<1	<1	<1	<1	<1	<1
Plasma cortisol at 0800 h, post 1 mg dexamethasone orally at 2400 h (nmol/liter; N < 50)	169	681	280	ND	ND	ND
Urinary free cortisol after 2 mg dexamethasone orally/d for 2 d (nmol/d)	ND[a]	ND[a]	112 (N < 28)	418 (N < 28)	1382 (N < 28)	218 (N < 28)
Screening tests, inducing a positive cortisol response[b]	Cosyntropin: +395% LVP: +403% Cisapride: +65%	Cosyntropin: +807% GnRH: +114% LH: +500% MCP: +157% Cisapride: +378%	Cosyntropin: +413% Cisapride: +509%	Cosyntropin: +170% Upright posture: +44% Cisapride: +49%	Cosyntropin: +220% Upright posture: +62% LVP: +52% Cisapride: +70%	Cosyntropin: +206% Upright posture: +29% GnRH: +64% Cisapride: +50%

N, Normal range; ND, not done; MCP, metoclopramide.

[a] In patients P1 and P2, plasma cortisol levels were not suppressed by 4 mg dexamethasone iv.

[b] The amplitude of the peak of plasma cortisol is expressed as percentage of basal values.

1 mg terlipressin [a precursor of lysine vasopressin (LVP), Glypressine, Ferring Pharmaceuticals Ltd., Gentilly, France], or 10 IU arginine vasopressin im. In addition, all patients underwent a cisapride stimulation test consisting of an oral administration of 10 mg cisapride (Prepulsid, Laboratoires Janssen-Cilag, Boulogne-Billancourt, France) and plasma cortisol measurements at 0, 60, and 120 min. In patient 2, the adrenal sensitivity to 5-HT$_4$ receptor agonists was also investigated with 10 mg metoclopramide orally (Maxeran, Aventis Pharma Inc., Laval, Québec, Canada). Plasma cortisol was measured by using commercial immunofluorometric kits and ACTH by immunoradiometric assay (Nichols Institute Diagnostics, San Juan Capistrano, CA). For all tests, a positive cortisol response was arbitrarily defined as a 25% increase in plasma cortisol. The tests inducing a positive plasma cortisol response are reported for the six patients in Table 1. In all cases, cisapride induced a significant increase in plasma cortisol levels. In addition, plasma ACTH levels remained undetectable throughout the study in all patients (data not shown).

Quantitative expression of the 5-HT$_4$ receptor

All patients with AIMAH underwent bilateral adrenalectomy, except patient 2 in which a biopsy of the left adrenal gland was obtained. Fragments of the tissue nodules were immediately stored at −80 C until RNA extraction. Normal adrenal glands were obtained from seven patients undergoing expanded nephrectomy for kidney cancer. The protocol of collection of the tissues and the experimental procedures were approved by the regional and/or institutional ethics committees, and written informed consent was obtained from all subjects.

Amplification of the 5-HT$_4$ receptor by RT-PCR. Total RNA from the six hyperplasias and seven normal adrenal glands was extracted by the acid guanidium-thiocyanate-phenol-chloroform procedure by using Tri Reagent (Sigma, St. Louis, MO). The concentration of total RNA was determined by measuring the optical density at 260 nm. Total RNA (1 μg) from each tissue was converted to single stranded cDNA using SuperScript II from Life Technologies, Inc. (Eragny, France) with oligo $(dT)_{12–18}$ primer (0.5 μg/ml). Amplification of the domain common to all 5-HT$_4$ receptor isoforms was performed by PCR with the primers S1 and As1 (Table 2). S1 and As1 primers were designed in exon 4 [nucleotides (nts) 697–717] and exon 5 (nts 1020–1043), respectively, of the human 5-HT$_4$ receptor gene (Fig. 1). Two other primers (5′-TGCTGAGTAYGTCGTGGAGTC-3′ and 5′-TTGGTGGTGCAGGAKGCATTGC-3′), corresponding to bases 297–317 and 467–488, respectively, of the cloned glyceraldehyde-3-phosphate dehydrogenase sequence (GenBank accession no. M17701) were used for semiquantitation of reverse transcribed mRNAs. The PCRs were performed for 40 cycles (94 C, 40 sec; 50 C, 60 sec; 72 C, 90 sec). The amplified products were analyzed in 1.5% agarose gels, blotted on a nylon membrane, and hybridized with a [^{32}P]ATP-labeled internal oligonucleotide S2 (Table 2, nts 873–893). In addition, PCR products were subcloned into pGEM-T (Promega Corp., Charbonnières, France) and sequenced using the Thermosequenase kit (Amersham, Orsay, France) on a Li-Cor 4200L DNA sequencer (ScienceTec, Les Ulis, France) using fluorescent T7 and T3 primers (MWG-Biotech, Courtaboeuf, France).

TaqMan PCR. Real-time RT-PCR assays were carried out as described by Fink *et al.* (15) to quantify 5-HT$_4$ receptor mRNA in both hyperplastic and normal adrenal cortices. The primers and fluorogenic TaqMan probe used for these experiments hybridized to all 5-HT$_4$ receptor splice variants (5-HT$_{4pan}$; Table 3). Briefly, 1 μg of total RNA was reverse transcribed in triplicate using Maloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.) and oligo dT primer as described above. The cDNA prepared was diluted and aliquoted into microtiter plates. For each 25-μl TaqMan reaction, 5 μl cDNA was mixed with 1 μl water, 12.5 μl TaqMan Universal PCR Master Mix 2X (Applied Biosystem, 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 sec and 60 C for 1 min. In addition, parallel assays using the same cDNA pools were performed using primers and probe to the housekeeping gene porphobilinogen deaminase (PBGD; Table 3). Quantitative RT-PCR was performed using an ABI Prism 7700 sequence detector system (Applied Biosystem) and analyzed using relative expression to PBGD, as previously described (15). Briefly, the level of expression in each sample was

TABLE 2. RT-PCR analysis of 5-HT$_4$ receptor isoform mRNAs

Primer	GenBank accession no.	Sequence (5′-3′)
S**1**	Y10437	CGGGCAGGAGCCTCCTCCGAGAG
As**1**	Y10437	CAAGGGACAGTCTGGCCCAGAATG
Fw**h**	AJ243213	GAAAGGAGTCTAAACCAAGGCCTGG
Rev**h**	AJ243213	CATGTGTGGATCCATTAATGGTTGTGG
Fw**1**	Y10437	AATTGATTTGATAGAAAAGAGGAAG
Rev**a**	Y08756	GTATGGGCAGTTTCTCGAGTTCCTGATGATG
Rev**b**	Y12505	GAAGTTGCTGGCGGGTGACACTGACTCTC
Rev**c**	Y12506	GGCATTAGGATGGTTTGGTCAATC
Rev**d**	Y12507	CATCCAATGAATTTATTTGATAACTTCAG
Rev**e**	AJ011371	CAGACGGGAACAGGTCTATTGCAGAAG
Rev**f**	AJ243213	TTAGACGGGAACAGGTCTTAGTACATG
Rev**g**	AJ243213	CAGAAGAGCAGGAGGAAGCTGGAGACAG
Rev**n**	AJ278982	ACTGGAGCATTACCCCTTCTGAG
S**2**	Y10437	CCCTGGGCAGGTGTGGACTGC

Oligonucleotide sequences for sense and antisense primers are shown.

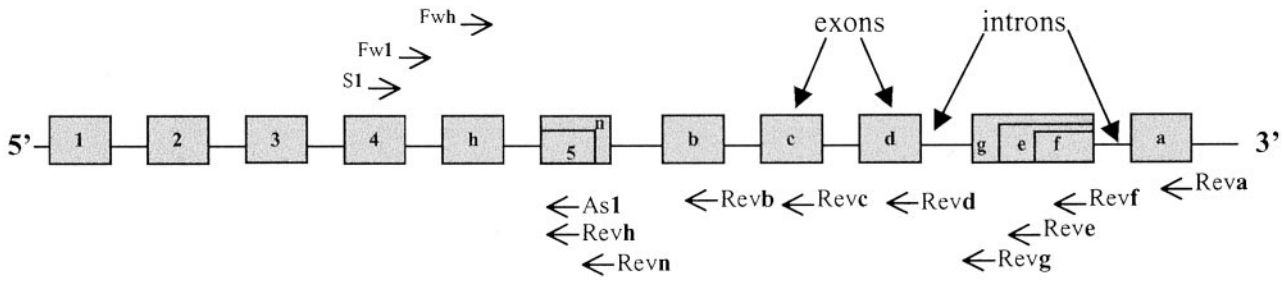

FIG. 1. Schematic representation of the human 5-HT$_4$ receptor gene showing the position of the primers used to amplify the 5-HT$_4$ receptor transcripts.

TABLE 3. TaqMan PCR analysis of 5-HT$_4$ receptor mRNA

cDNA	GenBank accession no.	Primer	Sequence (5′-3′)
5-HT$_{4pan}$	Y10437	s	AACGGCATCGATTTTTCACC
		as	GTTCCTATAGACCAAAGGCTGGC
		p	TGCTGCATTTCTCTGGATAGGTATTACGCC
PBGD	X04217	s	CTGCAACGGCGGAAGAA
		as	AGCTGGCTCTTGCGGGTAC
		p	CCCAAAGATGAGAGTGATTCGCGTGG

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

normalized by dividing copies per nanogram total RNA of 5-HT$_4$ receptor gene by copies per nanogram total RNA of the housekeeping gene PBGD, and expressed as a percentage. This mode of calculation allows to correct for both RNA quality and quantity. In each group of tissues, *i.e.* normal and hyperplastic glands, data are presented as mean ± SEM.

Characterization of 5-HT$_4$ receptor isoforms by RT-PCR

Total RNA was extracted and reverse transcribed as described above. Amplification of the cDNAs encoding the different 5-HT$_4$ receptor C-terminal splice variants was performed by PCR using primer S1 (Table 2), which hybridizes to all 5-HT$_4$ receptor messages and splice variant-specific reverse primers (Table 2 and Fig. 1). The 5-HT$_{4(h)}$ variant was amplified using the forward primer Fwh, which is specific for cDNAs containing the 5-HT$_{4(h)}$ exon, and the reverse primer Revh, which hybridizes to all 5-HT$_4$ receptor messages. 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, Inc.), DNA Polymerase buffer (Life Technologies, Inc.), 1.5 mM $MgCl_2$, 0.4 mM deoxynucleoside triphosphate, and 20 pmol of each primer. The PCRs were performed for 40 cycles (94 C, 40 sec; 50 C, 60 sec; 72 C, 90 sec). For each tissue, the PCR products were analyzed in 1.5% agarose gels, blotted on a nylon membrane, and hybridized with the [^{32}P]ATP-labeled oligonucleotide S2. All PCR products were subcloned and sequenced as described above. In addition, the full-length cDNAs encoding the isoforms expressed by hyperplasias P2 and P5 were amplified by PCR using the primer exon 1 (5′-ATGGACAAACTTCATGCTAATGTG-3′) corresponding to the first 24 nucleotides of the cloned 5-HT$_4$ receptor sequence (GenBank accession no. Y10437) and splice variant specific reverse primers (Table 2 and Fig. 1). PCR products of the expected sizes were subcloned into pGEM-T and sequenced. For each 5-HT$_4$ receptor splice variant, the full-length cDNA sequence was determined from six clones.

Results

Quantitative expression of 5-HT$_4$ receptors in normal adrenal glands and hyperplasias

The occurrence of 5-HT$_4$ receptor mRNA in adrenocortical hyperplasias and normal adrenal cortex was investigated by RT-PCR amplification using oligonucleotides hybridizing to all receptor variants. 5-HT$_4$ receptor PCR products were detected in all of the tissues studied (Fig. 2). Individual bands obtained from hyperplasia and normal adrenal tissue reverse-transcribed RNAs were excised, ligated into pGEM-T, and sequenced. All sequences corresponded to the published sequence of the 5-HT$_4$ receptor cDNA (data not shown). The quantitative expression of 5-HT$_4$ receptor mRNA by the tissues was then determined by TaqMan PCR. When expressed as arbitrary units normalized to PBGD, 5-HT$_4$ receptor expression ranged from 1.55% to 33.9% (mean, 13.1% ± 5.39%) in hyperplasias *vs.* 0.48% to 3.72% (mean, 2.13% ± 0.46%) in normal adrenal glands (Fig. 3). Two hyperplasias (P2 and P5) had levels of 5-HT$_4$ receptor expression similar to those of normal tissues, *i.e.* 1.65% and 1.55%, respectively. In the four remaining hyperplasias, 5-HT$_4$ receptor expression was 3 to 16 times higher than in normal glands (Fig. 3).

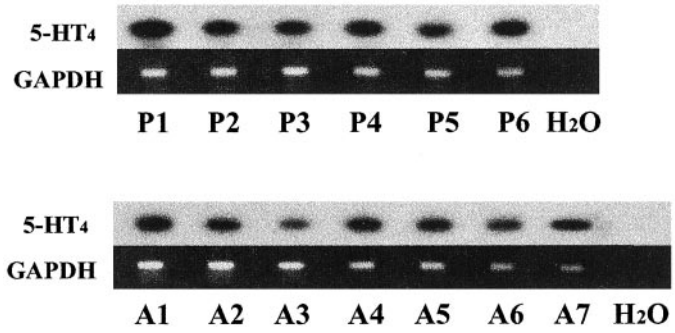

FIG. 2. RT-PCR analysis of total 5-HT$_4$ receptor mRNAs in the adrenal hyperplasias removed from patients 1–6 (P1–P6) and seven normal human adrenal cortices (A1–A7). The primers used (S1 and As1; see Table 2 for oligonucleotide sequences) were designed to amplify all 5-HT$_4$ receptor splice variants.

Characterization of 5-HT$_4$ receptor isoforms by RT-PCR

5-HT$_4$ receptor isoforms were characterized by RT-PCR amplification in both hyperplastic adrenal cortex and normal adrenal glands. In the two types of tissues, the profile of expression of 5-HT$_4$ receptor variants was variable from one sample to the other (Table 4). Hyperplastic tissues were found to express isoforms a–d, f, h, and n. Five hyperplasias expressed isoform a, four expressed isoform n, three expressed isoform b, two expressed isoform d, and one expressed isoform h. Isoforms c and f were expressed by all hyperplasias, whereas isoforms e and g were not detected in any hyperplastic tissue. The 5-HT$_4$ receptor variants present in hyperplasias P2 and P5, which did not overexpress the 5-HT$_4$ receptor, *i.e.* variants a–d and f, could also be observed in normal adrenal glands (Fig. 4). Isoform n was detected in all normal adrenal tissues, whereas isoforms a, c, and f were characterized in six of the seven glands studied. In addition, five adrenal glands expressed isoform b, and one expressed isoform d. None of the normal adrenal tissues expressed isoforms e, g, and h. Finally, the cDNAs encoding isoforms a–d and f in hyperplasias P2 and P5 were subcloned and full-length sequenced to detect a possible mutation causing gain-of-function of the 5-HT$_4$ receptor. All sequences were identical to those of the coding regions of the published 5-HT$_4$ receptor gene (data not shown).

Discussion

Previous studies have shown that, in some patients with Cushing's syndrome secondary to AIMAH, cortisol secretion

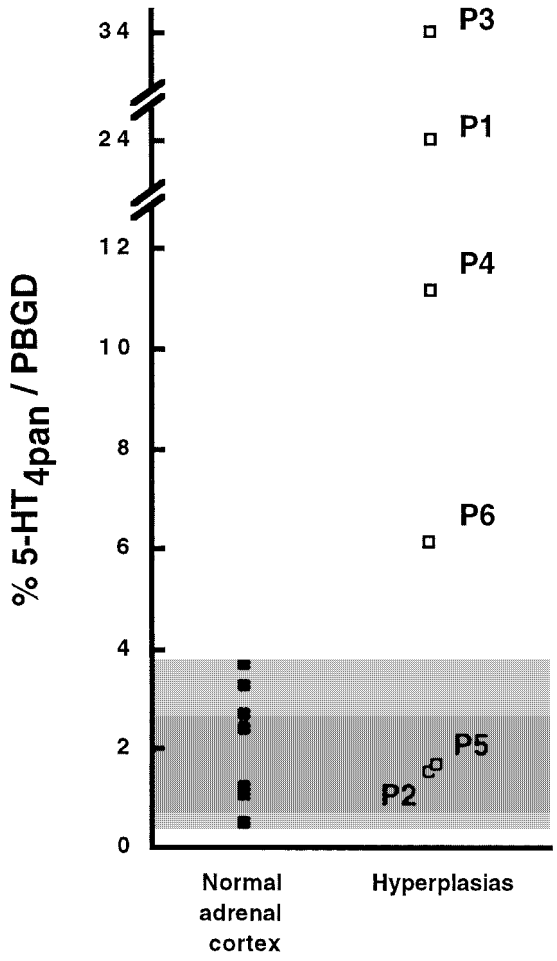

FIG. 3. Expression levels of 5-HT$_4$ receptor mRNAs in seven normal human adrenal cortices and the six adrenal hyperplasias (P1–P6). TaqMan RT-PCR analysis was performed with primers and a fluorogenic probe that recognized all 5-HT$_4$ splice variants (5-HT$_{4pan}$; see Table 3 for oligonucleotide sequences). 5-HT$_4$ receptor mRNA expression was expressed as arbitrary units normalized to PBGD. The *shaded area* corresponds to the range of variation of expression levels observed in normal tissues.

is stimulated by oral administration of the 5-HT$_4$ receptor agonists cisapride and/or metoclopramide (11–13). In contrast, cisapride administration to healthy volunteers has no influence on plasma cortisol levels (10), suggesting that the 5-HT$_4$ receptor, which is physiologically expressed by the zona fasciculata of the adrenal cortex (6), can be overexpressed or more efficiently coupled to adenylyl cyclase in adrenocortical hyperplastic tissues. Our results show that cisapride/metoclopramide-responsive AIMAHs causing subclinical and/or patent Cushing's syndrome express the 5-HT$_4$ receptor in very much the same way as normal adrenal glands. They also indicate that expression of the 5-HT$_4$ receptor is markedly increased in four of the six hyperplasias studied. In these tissues, 5-HT$_4$ receptor expression was 3 to 16 times higher than in normal adrenal cortices. Overexpression of the 5-HT$_4$ receptor likely accounts for the abnormal *in vivo* response to cisapride because it has been shown that, in COS-7 cells transfected with 5-HT$_4$ receptors, both basal and 5-HT$_4$ receptor agonist-stimulated cAMP productions

TABLE 4. RT-PCR analysis of 5-HT$_4$ receptor isoform (a–h, n) mRNAs in the six hyperplasias removed from patients 1 to 6 (P1–P6) and seven normal adrenal cortices (A1–A7)

	a	b	c	d	e	f	g	h	n
P1	+	−	+	−	−	+	−	−	+
P2	−	+	+	+	−	+	−	−	−
P3	+	−	+	−	−	+	−	−	+
P4	+	−	+	−	−	+	−	−	+
P5	+	+	+	+	−	+	−	−	−
P6	+	−	+	−	−	+	−	+	+
A1	+	+	+	−	−	+	−	−	+
A2	+	+	+	+	−	+	−	−	+
A3	+	−	+	−	−	+	−	−	+
A4	−	−	−	−	−	−	−	−	+
A5	+	+	+	−	−	+	−	−	+
A6	+	+	+	−	−	+	−	−	+
A7	+	+	+	−	−	+	−	−	+

RT-PCR amplification used pairs of primers specific for the different splice variants. See Table 2 for oligonucleotide sequences.

increase as a function of receptor density (16). 5-HT$_4$ receptor overexpression may also be involved in the development of adrenal hyperplasia and increased cortisol production. Indeed, it is well established that, in adrenocortical cells, activation of the cAMP pathway enhances the mitogenic activity and stimulates steroidogenesis (17–20). The pathophysiological mechanisms responsible for the overexpression of the 5-HT$_4$ receptor in hyperplasias are currently unknown. As previously proposed by Lacroix *et al.* (11), it is possible that a mutational event modifying the expression of the 5-HT$_4$ receptor may have occurred at an early stage of adrenal differentiation during embryogenesis. In our series of AIMAHs, 5-HT$_4$ receptor overexpression was associated with abnormal expression of other membrane receptors, including LH/human chorionic gonadotropin and V$_1$-vasopressin receptors, suggesting the occurrence of a mutation in a transcription factor controlling the expression of multiple receptors in the adrenocortical tissue.

In two hyperplasias (P2 and P5), the level of 5-HT$_4$ receptor expression was similar to that of normal adrenal glands. Owing to the fact that 5-HT$_4$ receptor variants may exhibit different coupling efficiencies (21), we thus hypothesized that, in some cases, the *in vivo* responsiveness of hyperplastic adrenal glands to cisapride and/or metoclopramide could be ascribed to expression of 5-HT$_4$ receptor isoforms that differ from those present in the normal adrenal cortex. We found that the 5-HT$_4$ receptor variants expressed by hyperplasias P2 and P5, *i.e.* variants a–d and f, could also be detected in normal adrenocortical tissue, indicating that the pathological *in vivo* cortisol response to cisapride was not attributable to the presence of ectopic 5-HT$_4$ receptor isoforms in these two tissues. Isoform h was characterized in one hyperplasia overexpressing the 5-HT$_4$ receptor (P6) but not in the series of normal adrenal tissues. However, several observations indicate that the enhanced responsiveness of the hyperplastic tissue to cisapride could not be accounted for by aberrant expression of this isoform: 1) the potencies and efficacies of cisapride to stimulate cAMP formation in COS-7 cells transfected with either the human 5-HT$_{4(h)}$ isoform or the C-terminal splice variants, *i.e.* the 5-HT$_{4(a)}$ and 5-HT$_{4(b)}$ isoforms, are similar (5); 2) a study of 5-HT$_{4(h)}$ mRNA distribution in human tissues has shown that isoform h can

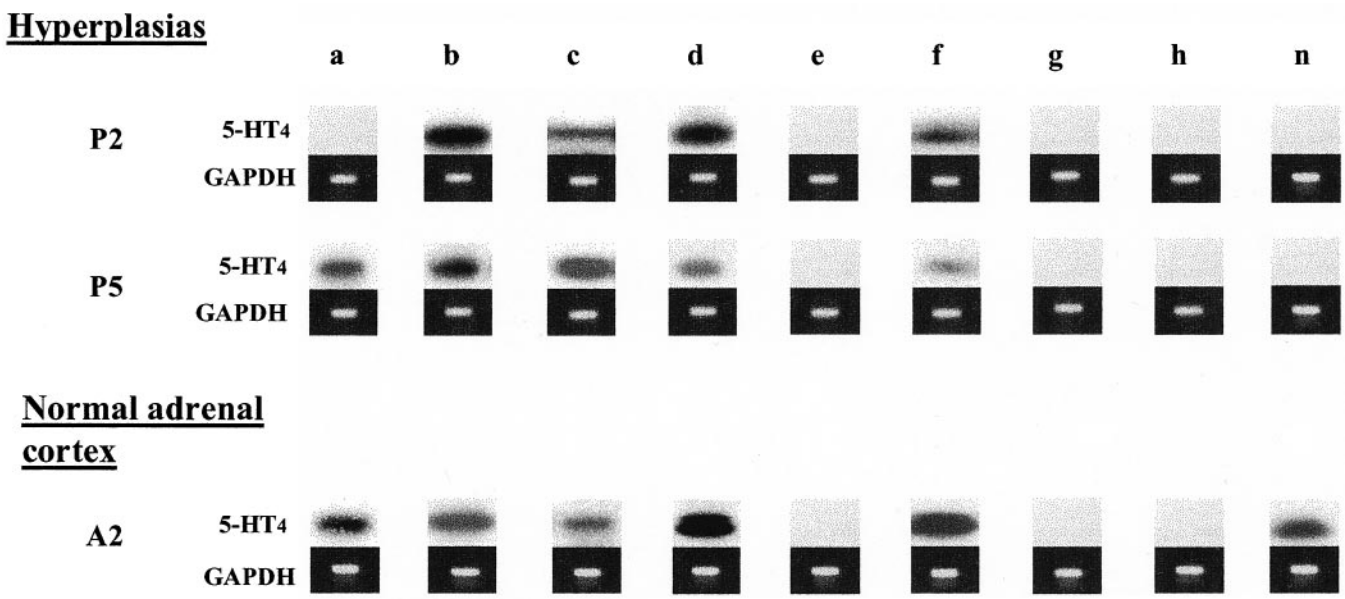

FIG. 4. RT-PCR analysis of 5-HT$_4$ receptor isoforms a–h and n in hyperplasias P2 and P5 and a normal adrenal cortex (A2). RT-PCR amplification used pairs of primers specific for the different splice variants. See Table 2 for oligonucleotide sequences.

also be expressed in the normal adrenal gland (5). Recent studies have shown that G protein-coupled receptors can form homodimers and/or heterodimers that modulate receptor function (22, 23). It is therefore possible that differences in the expression profiles of 5-HT$_4$ receptor splice variants could induce a functional variability in 5-HT$_4$ receptor-mediated tissue responses. In particular, an abnormal expression profile of 5-HT$_4$ receptor isoforms could be involved in the pathological cortisol response to cisapride in some patients with AIMAH. Consistent with this hypothesis, the expression profiles of the 5-HT$_4$ receptor splice variants in hyperplasias 2 and 5, *i.e.* a−/b+/c+/d+/e−/f+/g−/h−/n− and a+/b+/c+/d+/e−/f+/g−/h−/n−, respectively, were not observed in the series of normal adrenal cortices.

Gain-of-function mutations of G protein-coupled receptors leading to increased cAMP production and cellular proliferation have been described in various endocrine diseases, including familial neonatal hyperthyroidism and testotoxicosis (24, 25). In addition, site-directed mutagenesis studies have shown that mutations occurring within the third intracellular loop of the 5-HT$_4$ receptor, such as mutation A258L, increase the constitutive activity of the receptor as well as its affinity for 5-HT and its coupling efficiency (16). We have thus examined whether the enhanced sensitivity of the hyperplastic adrenal tissues P2 and P5 to 5-HT$_4$ receptor agonists could be ascribed to the occurrence of somatic point mutations causing gain of function of 5-HT$_4$ receptors within the adrenal tissue. Our data revealed that, in the hyperplastic adrenal glands removed from patients 2 and 5, the sequences of the cDNAs encoding the isoforms expressed by the tissues (a–d and f) were identical to those of the coding regions of the published human 5-HT$_4$ receptor gene. This observation indicates that the cellular defect leading to enhanced adrenal sensitivity to 5-HT and hyperplasia cannot be accounted for by an activating mutation of the 5-HT$_4$ receptor.

In conclusion, our results show for the first time an overexpression of the eutopic 5-HT$_4$ receptor in AIMAHs responsive *in vivo* to cisapride administration. In some cases however, the level of expression of the receptor is similar to that of normal adrenal tissue. In this situation, the enhanced sensitivity of the tissue to cisapride is not due to ectopic expression of 5-HT$_4$ receptor isoforms or to the occurrence of somatic gain-of-function mutation of the receptor.

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