0021-972X/04/$15.00/0
Printed in U.S.A.

The Journal of Clinical Endocrinology & Metabolism 89(9):4493–4502

doi: 10.1210/jc.2003-031746

Dopamine Receptor Expression and Function in Human Normal Adrenal Gland and Adrenal Tumors

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Dopamine is known to play a role in the modulation of aldosterone and catecholamine secretion from the adrenal gland, where dopamine receptors (DR), in particular the DR type 2 (D_2), have been found to be expressed. DR expression has also been demonstrated in some types of benign adrenal tumors.

The aims of the current study were to evaluate DR expression and D_2 localization in the normal adrenal gland and in different types of benign and malignant adrenal tumors, as well as to evaluate the *in vitro* effects of the dopamine agonists bromocriptine and cabergoline on hormone secretion in nontumoral adrenal cells.

Adrenal tissues from 25 patients, subjected to adrenal surgery for different diseases, were studied. These included three normal adrenals; five adrenal hyperplasias; four aldosterone-secreting, two cortisol-secreting, and two clinically nonfunctioning adrenal adenomas; two aldosterone-secreting, two cortisol-secreting, and two androgen-secreting adrenal carcinomas; and three pheochromocytomas. In all tissues, DR and D_2 isoform (D_{2long} and D_{2short}) expression was evaluated by RT-PCR. D_2 localization was also evaluated by immunohistochemistry using a specific polyclonal antibody, whereas D_2-like receptor expression was evaluated by receptor-ligand binding study, using the radiolabeled D_2 analog ^{125}I-epidepride. The effects of bromocriptine and cabergoline on baseline and ACTH and/or angiotensin II-stimulated aldosterone, cortisol, and androstenedione secretion were evaluated in cell cultures derived from five different adrenal hyperplasia.

At RT-PCR, both D_1-like and D_2-like receptors were expressed in all normal and hyperplastic adrenals. D_2 and D_4 were expressed in aldosterone- and cortisol-secreting adenomas, cortisol-secreting carcinomas, and clinically nonfunctioning adenomas, whereas no DR was expressed in aldosterone- and androgen-secreting carcinomas. D_2, D_4, and D_5 were expressed in pheochromocytomas. In all D_2-positive tissues, both D_2 isoforms were expressed, with the exception of one case of aldosterone-secreting adenoma and the cortisol-secreting carcinomas, in which only the D_{2long} isoform was expressed. D_2-like receptor expression was confirmed at receptor-ligand binding study. At immunohistochemistry, D_2 was mainly localized in the zona glomerulosa and reticularis of the adrenal cortex and, to a lesser extent, in the zona fasciculata and medulla of normal and hyperplastic adrenal tissue. In the positive tumors, D_2 was localized in the tumoral cells. At the *in vitro* study, a significant inhibition of both baseline and ACTH-stimulated aldosterone secretion was found after high-dose cabergoline, but not bromocriptine, administration; and a significant inhibition of angiotensin-II-stimulated aldosterone secretion was found after both bromocriptine and cabergoline administration in the adrenal hyperplasias.

In conclusion, the current study demonstrated that both D_1-like and D_2-like receptors are expressed in the normal adrenal gland and in a percentage of adrenal adenomas or carcinomas. Bromocriptine and cabergoline induce only a minor inhibition of the secretion of adrenal hormones in the nontumoral adrenal gland *in vitro*, not excluding, however, the possible effective use of dopamine agonists *in vivo* in the treatment of adrenal tumors. (*J Clin Endocrinol Metab* 89: 4493–4502, 2004)

DOPAMINE IS THE predominant catecholamine neurotransmitter in the human central nervous system, but plays multiple roles in the periphery, as a modulator of cardiovascular and renal function and endocrine regulation (1). The various actions of dopamine are mediated by five specific receptors (D_1–D_5), which can be subdivided in two different receptor families on the basis of their biochemical and pharmacological characteristics: D_1-like (including the D_1 and D_5) and D_2-like (including the D_2, D_3, and D_4 receptor) (1). D_2 receptor exists in two different isoforms, the long (D_{2long}) and short (D_{2short}) isoforms (1). The different dopamine receptor (DR) subtypes have a differential distribution and play different roles in various organs and tissues (1).

The dopaminergic system is known to regulate the renin-angiotensin-aldosterone system (2). D_1-like receptors are expressed in the renal juxtaglomerular apparatus, where D_1-selective dopamine agonists stimulate renin secretion (3, 4). Both D_1- and D_2-like receptors have been reported to be expressed in the adrenal medulla, where they modulate catecholamine release (5–7). Conversely, DR expression and function in the normal adrenal cortex are still a matter of debate. Indeed, the administration of the D_2 antagonist

Abbreviations: Bmax, Maximal binding; D_2, DR type 2; DR, dopamine receptor(s); HPRT, hypoxantine ribosyl transferase; IHC, immunohistochemistry; ISH, *in situ* hybridization; Kd, dissociation coefficient; RLBS, receptor-ligand binding study; TBS-T, Tris buffered saline-Tween; WGA, wheat germ agglutinin.

JCEM is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.

metoclopramide was shown to directly increase plasma aldosterone levels (8), without the mediation of any modulator of aldosterone secretion (9, 10). However, dopamine or dopamine agonists, such as bromocriptine, did not modify basal plasma aldosterone levels (11), suggesting that aldosterone production is under maximal tonic dopaminergic inhibition. The presence of D_2-like receptors in the adrenal cortex has been evaluated by binding studies, which demonstrated their localization mainly in the zona glomerulosa of the adrenal cortex (12). Moreover, they were demonstrated to mediate the inhibition of angiotensin-stimulated, but not basal and ACTH-stimulated, aldosterone secretion by dopamine agonists (13). Recently, both D_2 and D_4 receptors have been demonstrated, by molecular studies, to be heterogeneously expressed in all three zones of the adrenal cortex (14).

DR expression has not been extensively evaluated in adrenal tumors. D_2 and D_4 receptors have been recently demonstrated, by RT-PCR and *in situ* hybridization (ISH), in pheochromocytomas, the tumors deriving from adrenal medulla, and in benign aldosterone-producing tumors (6, 14). However, no study has ever evaluated DR expression in benign cortisol- or sex hormone-producing or clinically nonfunctioning tumors as well as in malignant cortical adrenal tumors.

The aim of the current study was 3-fold: 1) to evaluate DR and D_2 isoform expression by RT-PCR, and D_2-like receptor expression by receptor-ligand binding study (RLBS) as well, in normal adrenal and adrenal hyperplasia and tumors; 2) to evaluate the localization of D_2 receptor expression by immunohistochemistry (IHC) in normal adrenal and adrenal hyperplasia and tumors; and 3) to evaluate the *in vitro* effect of the dopamine agonists bromocriptine and cabergoline on baseline and ACTH and/or angiotensin II-stimulated aldosterone, cortisol, and androstenedione concentration on cultured adrenal cells deriving from five different hyperplastic adrenals.

Subjects and Methods

Subjects

The adrenal samples were surgically removed from 25 patients (14 males, 11 females; age range, 34–66 yr; mean, 48.5 ± 0.7; median, 48.0), operated for renal or adrenal diseases, after their informed consent had been obtained. Three of the 25 patients were subjected to monolateral nephrectomy for renal carcinomas, eight to adrenal tumorectomy, nine to monolateral adrenalectomy, and the remaining five to bilateral adrenalectomy for adrenal diseases. The adrenal case load included three normal adrenals (deriving from the patients subjected to nephrectomy), five adrenal hyperplasias (deriving from patients with ACTH-dependent Cushing's syndrome), four aldosterone-secreting adrenal adenomas and two aldosterone-secreting adrenal carcinomas (deriving from patients with Conn's syndrome), two cortisol-secreting adrenal adenomas and two cortisol-secreting adrenal carcinomas (deriving from patients with ACTH-independent Cushing's syndrome), two androgen-secreting carcinoma (deriving from two female patients with virilizing syndrome), two clinically nonfunctioning adrenal adenoma (incidentalomas), and three pheochromocytomas. The diagnosis were performed on the basis of clinical, biochemical, hormonal, radiological, and pathological features of the respective cases.

Samples

Adrenal specimens were obtained, at the time of surgery, from the 25 patients. Samples of these specimens were taken fresh directly at the operation. A sample was fixed in 10% paraformaldehyde overnight and then embedded in paraffin for the IHC study. An additional sample was quickly frozen on dry ice and stored in a freezer at −80 C for RT-PCR and/or RLBS. In selected cases, a fresh sample was also used for the establishment of adrenal primary culture.

Study design

In all 25 cases, DR expression was evaluated by RT-PCR, whereas D_2-like receptor expression was evaluated by RLBS on adrenal cell membrane preparations in one case of normal adrenal, one case of adrenal hyperplasia, one case of cortisol-producing adenoma, and one case of cortisol-producing carcinoma. D_2 receptor expression was evaluated also by IHC in all 25 cases. In the three cases of adrenal hyperplasia, the *in vitro* effect of dopamine agonists bromocriptine and cabergoline was also evaluated. In two additional cases of adrenal hyperplasias, the effect of bromocriptine and cabergoline on angiotensin II-stimulated and ACTH-stimulated aldosterone secretion was also evaluated. The protocol was in accordance with the Helsinki Doctrine on Human Experimentation, and it was approved by the local ethical committee.

RLBS

The frozen tissue samples were used for the membrane RLBS. The methodology of membrane isolation has been previously described (15). Briefly, the tissue samples were homogenized in a Polytron tissue homogenizer in ice-cold homogenization buffer [50 mM Tris-HCl (pH 7.7), 0.32 M sucrose, 1 mM phenylmethylsulfonylfluoride, 0.25 mM $CaCl_2$] and centrifuged at 600 × *g* for 5 min at 4 C to remove nuclei and unbroken cells. The supernatant was then collected and centrifuged at 14,000 × *g* for 30 min at 4 C in an Eppendorf microcentrifuge. The pellet was washed once and resuspended in the same buffer. The protein content of membrane samples was determined by a spectrophotometric method. Membrane preparations (corresponding to 30–60 μg protein) of tissue sample were resuspended in binding buffer [50 mM Tris-HCl (pH 7.7), 120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$ $2H_2O$, 1 mM $MgCl_2$ $6H_2O$, 0.1% ascorbic acid] and incubated in a total vol of 100 μl at room temperature for 60 min with increasing concentrations of the D_2 analog (D_2 antagonist) ^{125}I-epidepride (Radiopharmaka, Seibersdorf, Austria) with and without excess (1 μM) of an unlabeled D_2 agonist cabergoline. After the incubation, 1 ml ice-cold Tris buffer was added to the reaction mixture, and membrane-bound radioactivity was separated from unbound by centrifugation during 2 min at 14,000 × *g* in Eppendorf microcentrifuges. The remaining pellet was washed twice in ice-cold Tris buffer, and the final pellet was counted in a γ-counter. Specific binding was taken to be total binding minus binding in the presence of 1 μM unlabeled cabergoline. Scatchard analysis was performed to measure the maximal binding (Bmax) and the dissociation coefficient (Kd) of the binding. Rat brain basal ganglia were used as controls in all the experiments. The experiment was performed three times for each case.

IHC

The formalin-fixed and paraffin-embedded tissue samples were used for the IHC. The methodology of immunohistochemistry has been previously described (16). The tissue samples were cut into 5-μm-thick sections. The sections were deparaffinized, dehydrated, exposed to microwave heating (in citric acid buffer) at 100 C for 15 min, rinsed in tap water followed by PBS, and subsequently incubated for 15 min in normal goat serum (1:10 dilution in PBS + 5% BSA). The sections were then incubated overnight at 4 C with a rabbit antihuman D_2 receptor polyclonal antibody (Chemicon International, Temecula, CA) at a dilution of 1:500. A standard streptavidin-biotinylated-alkaline phosphatase or -peroxidase complex (ABC kit, Biogenix, San Ramon, CA) was used according to the manufacturer's recommendation to visualize the bound antibodies. Negative controls for the immunohistochemistry included: 1) omission of the primary antibody; and 2) preabsorbtion of the antibodies with the respective immunizing receptor peptides (at a concentration of 100 nM). The immunostaining for the D_2 receptor and the negative controls were performed on sequential sections. Histological evaluation was performed on hematoxylin-eosin-stained sequential sections. Positive and negative controls were represented by dopa-

mine agonist-sensitive and dopamine agonist-resistant PRL-secreting pituitary tumors, respectively; their immunostaining for D_2 receptor was carried out in the same experiments of the adrenals. The sensitivity of the D_2 receptor antibody had been previously tested, performing an immunostaining with different dilutions of the antibodies (1:100, 1:250, 1:500, 1:1000) on sections from a PRL-secreting pituitary tumor, choosing the dilution with the maximum of specific and the minimum of aspecific staining. The specificity of the D_2 receptor antibody was tested by immunoblotting.

Immunoblotting

A frozen sample of a pheocromocytoma was used for the immunoblotting. Particularly, tumor tissue was suspended in a 10-vol ice-cold Tris-buffer (10 mM Tris-HCl, pH 7.6; 5 mM EDTA; 3 mM EGTA; 250 mM sucrose; 1 mM phenylmethylsulfonylfluoride; 10 μg/ml leupeptin; 10 μg/ml soybean-trypsin-inhibitor; 50 μg/ml bacitracin), homogenized with a Polytron homogenizer at 900 rpm for 10 strokes and then ultracentrifuged for 1 h at 4 C at 100,000 $\times$ *g*. Membrane pellet was solubilized in a lysis buffer (20 mM HEPES, 5 mM EDTA, 3 mM EGTA, 150 mM NaCl, 4 mg/ml dodecyl-β-D-maltoside) for 1 h at 4 C and then ultracentrifuged for 1 h at 4 C at 100,000 $\times$ *g*. Glycosylated proteins were purified from membrane pellet obtained after high-speed centrifugation, by wheat germ agglutinin (WGA) affinity chromatography: the pellet was resuspended in lysis buffer and cycled twice over a 0.5-ml WGA (Vector Laboratories, Burlingame, CA) column equilibrated with lysis buffer. The column was washed with lysis buffer and eluted with lysis buffer containing 3 mM *N*,*N*′,*N*″triacetyl-chitotriose (Sigma Chemical Co., St. Louis, MO). The protein-containing fractions was determined with the Bradford assay standardized with BSA, pooled, and stored at −80 C. Starting material and WGA-purified membrane proteins were denatured and fractionated under reducing conditions on 12.5% SDS-PAGE, then transferred electrophoretically to Hybond C-extra nitrocellulose membranes (Amersham Life Science, Oakville, Ontario, Canada). After transfer, nonspecific binding sites were blocked by treating membranes with Tris-buffered saline-Tween (TBS-T) containing 5% nonfat dried milk. After five washes with TBS-T, membranes were incubated for 16 h at 4 C with a 1:500 dilution of rabbit D_2 receptor polyclonal antibody (Chemicon International) in TBS-T containing 1% BSA. Membranes were washed five times with TBS-T and then incubated for 1 h at 22 C with 1:1000 dilution of horseradish peroxidase-linked antirabbit IgG (Amersham Life Science) and washed as before. The specificity of the antibody was confirmed preincubating the antibody with the respective immunizing receptor peptides (at a concentration of 100 nM). Immunoreactive bands were detected by a chemiluminescence detection system (ECL Western blot analysis system, Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK) according to manufacture's protocol. The immunoreactive bands were visualized by autoradiography after 0.5 min of exposure to Kodak Biomax film (Eastman Kodak Company, Rochester, NY). Because the antibody recognizes both the native and the denatured forms of D_2 receptor, bands of 110, 68, and 47 KDa may be visualized by Western blot. The expected band with the procedure used in our lab was the 68-kDa denatured form.

RT-PCR

The frozen tissue samples were used for the RT-PCR. The methodology for the isolation of mRNA and the synthesis and amplification of cDNA has been previously described (15). Messenger RNA was isolated using Dynabeads Oligo $(dT)_{25}$ (Dynal AS, Oslo, Norway) from a frozen tissue sample. The cells were lysed during 2 min on ice in a buffer containing 100 mM Tris-HCl (pH 8.0), 500 mM LiCl, 10 mM EDTA (pH 8.0), 1% LiDS, 5 mM dithiothreitol, and 5 U/100 μl ribonuclease inhibitor (HT Biotechnology Ltd., Cambridge, UK). The mixture was centrifugated at 14,000 rpm for 1 min to remove cell debris. To the supernatant, 100 μl prewashed Dynabeads Oligo $(dT)_{25}$ was added, and the mixture was incubated for 5 min on ice. Thereafter, the beads were collected with a magnet, washed three times with 10 mM Tris HCl (pH 8.0), 0.15 M LiCl, 1 mM EDTA, 0.1% LiDS, and once with a similar buffer from which LiDS was omitted. Messenger RNA was eluted from the beads in 50 μl of a 2-mM EDTA solution (pH 8.0) during 2 min at 65 C. To avoid contamination by genomic DNA, the isolated poly A^+ mRNA was subjected to a second purification by capturing the RNA on a fresh aliquot of prewashed Dynabeads Oligo $(dT)_{25}$ and washing the captured RNA as above described. cDNA was synthesized using the poly A^+ mRNA captured on the Dynabeads Oligo $(dT)_{25}$ in a buffer containing 50 mM Tris-HCl (pH 8.3), 100 mM KCl, 4 mM dithiothreitol, 10 mM $MgCl_2$, 1 mM of each deoxynucleotide triphosphate, 10 U ribonuclease inhibitor, and 2 U AMV Super Reverse Transcriptase (HT Biotechnology Ltd.) in a final vol of 20 μl. This mixture was incubated for 1 h at 42 C. One tenth from each cDNA library immobilized on the paramagnetic beads was used for each amplification. The amplification reaction mixture contained cDNA template, 0.5 U SuperTaq (HT Biotechnology Ltd.), 50 μM of each deoxynucleotide triphosphate (HT Biotechnology Ltd.), 5 pmol of each of a pair of oligonucleotide primers specific for human D_1–D_5 receptor subtypes or the hypoxantine ribosyl transferase (HPRT) in a buffer of 10 mM Tris-HCl (pH 9), 50 mM KCl, 2 mM $MgCl_2$, 0.01% (wt/vol) gelatin, 0.1% Triton X-100 in a final vol of 50 μl. The sequences of the primers for D_1–D_5 and HPRT are listed in Table 1. The PCR was carried out in a DNA thermal cycler with heated lid (Perkin-Elmer Cetus Instruments, Gouda, The Netherlands). After an initial denaturation at 94 C for 5 min, the samples were subjected to 40 cycles of denaturation at 94 C for 1 min, annealing for 2 min at 60 C, and extension for 1 min at 72 C. After a final extension for 10 min at 72 C, 10-μl aliquots of resulting PCR products were analyzed by electrophoresis on 1.5% agarose gels stained with ethidium bromide. Several controls were included in the RT-PCR experiments. To ascertain that no detectable genomic DNA was present in the poly A^+ mRNA preparation for two DR subtypes, D_1 and D_5, whose genes are intronless, the cDNA reactions were also performed without reverse transcriptase and amplified with each primer pair. Amplification of the cDNA samples with the HPRT specific primers served as positive control for the quality of cDNA. To exclude contamination of the PCR mixtures, the reactions were also performed in the absence of DNA template in parallel with cDNA samples. As a positive controls for the PCR of the DR subtypes and HPRT, 0.01 μg human brain cDNA was

TABLE 1. Specific oligonucleotide primers for dopamine receptor subtypes (D_1–D_5) and controls used in the RT-PCR study

Gene		Sequence (5′–3′)	Size of PCR product (bp)
Dopamine receptors			
D_1	Forward	AACACCTCTGCCATGGACG	616
	Reverse	TGATGGCCACAGGGATGTAA	
D_2	Forward	GCGGACAGACCCCACTACAA	521
	Reverse	AAGGGCACGTAGAAGGAGAC	
$D_{2\ short/long}$ isoforms	Forward	CCATGCTGTACAATACGCGCT	$D_{2\ long}$, 599; $D_{2\ short}$, 512
	Reverse	GGCAATCTTGGGGTGGTCTTT	
D_3	Forward	CCCGCCCACATGCCTACTAT	1106
	Reverse	GAAGGCTTTCCGGAACTCGAT	
D_4	Forward	CCCACCCCAGACTCCACC	259
	Reverse	GAACTCGGCGTTGAAGACAG	
D_5	Forward	ACCTGTGCGTCATCAGCGT	921
	Reverse	TGCGATCGAAAGGACCCTC	
HPRT	Forward	CAGGACTGAACGTCTTGCTC	417
	Reverse	CAAATCCAACAAAGTCTGGC	

amplified in parallel with the cDNA samples of each examined adrenal.

Cell culture and in vitro study

The fresh tissue samples were used for the establishment of cell cultures. The methodology of adrenal culture has been previously described (17). Adrenal tissue specimens were placed in Hanks' balanced salt solution (Invitrogen., Paisley, Scotland, UK), supplemented with human serum albumin 5% (Cealb; CLB, Amsterdam, The Netherlands), penicillin (10^5 U/liter), fungizone (0.5 mg/liter). After careful removal of blood clots, the specimens were minced and washed several times with the Hanks' balanced salt solution + human serum albumin. The minced tissue were enzymatically dissociated with collagenase. After removal of erythrocytes by centrifugation on a Ficoll density gradient, the adrenal cells were plated in 24-well plates (Corning, Cambridge, MA) in DMEM supplemented with 0.2% BSA. Viability of the cells was determined by trypan blue exclusion and was greater than 80% in every experiment. Cells were incubated at 37 C in a humid CO_2 incubator for 24 h with or without test substances in quadruplicate using 300,000 cells/ml. At the end of the incubation, 0.5 ml distilled water was added to each well, and the resulting suspension was collected and stored at −20 C for the measurement of aldosterone, cortisol, and androstenedione secretion. Baseline and ACTH-stimulated and/or angiotensin II-stimulated hormone secretion was evaluated without and with bromocriptine and cabergoline administration. ACTH (ACTH 1–24, Synachten, Ciba-Geigy, Basel, Switzerland) was added to the cell culture at the final concentration of 10^{-10} M. Angiotensin II (Clinalfa, Laufelfingen, Germany) was added to the cell culture at the concentration of 10^{-6} M. Bromocriptine (Novartis, Basel, Switzerland) and cabergoline (Pharmacia, Milan, Italy) were used as test substances and added to the cell cultures at the concentration of 10^{-12}, 10^{-10}, 10^{-9}, 10^{-8}, and 10^{-6} M for the experiment testing the baseline and ACTH-stimulated hormone secretion and at the concentration of 10^{-8} M for the experiments testing the angiotensin II-stimulated hormone secretion. Baseline aldosterone, cortisol, and androstenedione concentrations (measured in the medium from untreated cells or from cells with ACTH or angiotensin II alone) were compared with the respective hormone concentrations measured in the medium containing the different concentrations of bromocriptine and cabergoline.

Hormone assays

Aldosterone concentrations were measured by RIA using a commercially available kit from Biochem Immunosystem (Bologna, Italy); cortisol concentrations were measured by RIA using a commercially available kit from Diagnostic System Laboratories (Webster, TX); androstenedione concentrations were measured by a solid-phase, two-site chemiluminescent enzyme immunoradiometric assay from Diagnostic Products Corporation (Los Angeles, CA).

Statistical analysis

Data are expressed in mean ± SE The comparison between pre- and posttreatment values was performed by ANOVA followed by Bonferroni test for correction for multiple comparisons. Significance was set at 5%.

Results

RT-PCR study

Both D_1-like (D_1 and D_5) and D_2-like (D_2 and D_4) receptors were found to be expressed in all cases of normal and hyperplastic adrenals. D_2 and D_4 were consistently expressed in both aldosterone- and cortisol-secreting adenomas, which also expressed, though not in all cases, D_1 or D_5. However, among the aldosterone-secreting adenomas, D_2 was not expressed in one of the 4 (25%) cases. In addition, D_2 and D_4 were expressed in the 2 cases of cortisol-secreting carcinomas and in all the clinically nonfunctioning adrenal adenomas, whereas no DR was expressed in the aldosterone and androgen-secreting adrenal carcinomas. D_2, D_4 and D_5 were consistently expressed in the pheochromocytomas. In all cases positive for D_2, both D_2 isoforms were expressed, with the exception of the two cases of cortisol-secreting carcinomas and one of the three (33.3%) D_2-positive cases of aldosterone-secreting adenomas, in which only D_{2long} isoform was expressed. No D_3 receptor was found in any adrenal sample. The results of RT-PCR study are shown in Table 2.

RLBS study

Specific binding of ^{125}I-epidepride was found in all cases, including a normal adrenal, an adrenal hyperplasia, and a cortisol-secreting adrenal adenoma and carcinoma, with variable mean Bmax ranging from 22–40 fmol/mg protein and Kd ranging from 0.19–0.24 nM. The results of the RLBS are shown in Table 3. Two examples of a ^{125}I-epidepride binding in a normal adrenal and a cortisol-producing adrenal adenoma are shown in Fig. 1.

Immunoblotting

A specific band of the expected molecular weight of D_2 receptor was found at the immunoblot of glycoproteins derived from the pheochromocytoma (Fig. 2).

IHC study

Specific immunoreactivity for D_2 receptor was found in all cases of normal adrenal and adrenal hyperplasias. In these tissues, the immunostaining was localized in all three areas of the adrenal cortex and in the adrenal medulla. However, it was strongly positive in the zona glomerulosa and reticularis of the adrenal cortex, moderately positive in the adrenal medulla, and faintly positive in the zona fasciculata. Specific immunoreactivity for D_2 receptor was also found in three of the four (75%) aldosterone- (no. 9–11), the cortisol-producing, and the clinically nonfunctioning adrenal adenomas, as well as in the cortisol-producing carcinoma and in the pheochromocytomas, but not in one case of aldosterone-secreting adenoma (no. 12), the aldosterone-secreting, and the androgen-secreting adrenal carcinomas. A complete correspondence was found between the results of immunohistochemistry and RT-PCR study. Examples of D_2 receptor immunostaining in a normal adrenal, an adrenal hyperplasia, a cortisol-producing adrenal adenoma, and a cortisol-producing adrenal carcinoma are shown in Fig. 3.

In vitro study

A slight, but not significant, increase, at low dose (10^{-10} M), and decrease, at high dose (10^{-6} M), of both baseline and ACTH-stimulated cortisol and androstenedione secretion was found either after bromocriptine or cabergoline administration in the cell cultures deriving from the adrenal hyperplasias. Conversely, a significant stimulation at low dose and a significant inhibition at high dose of both baseline and ACTH-stimulated aldosterone secretion was found after cabergoline, but not bromocriptine, administration (Fig. 4). A significant decrease of angiotensin-II-stimulated aldosterone secretion at the dose of 10^{-8} M was found either after bro-

TABLE 2. Dopamine receptor expression in normal adrenal and adrenal tumors

Case	Diagnosis	RT-PCR study						
		D_1	D_2	D_{2s}/1	D_3	D_4	D_5	HPRT
1	Normal adrenal	+	+	Long/Short	−	+	+	+
2	Normal adrenal	+	+	Long/Short	−	+	+	+
3	Normal adrenal	+	+	Long/Short	−	+	+	+
4	Adrenal hyperplasia	+	+	Long/Short	−	+	+	+
5	Adrenal hyperplasia	+	+	Long/Short	−	+	+	+
6	Adrenal hyperplasia	+	+	Long/Short	−	+	+	+
7	Adrenal hyperplasia	+	+	Long/Short	−	+	+	+
8	Adrenal hyperplasia	+	+	Long/Short	−	+	+	+
9	Aldosterone-secreting adenoma	−	+	Long/Short	−	+	+	+
10	Aldosterone-secreting adenoma	−	+	Long/Short	−	+	−	+
11	Aldosterone secreting adenoma	−	+	Long	−	+	−	+
12	Aldosterone secreting adenoma	−	−	−	−	+	−	+
13	Cortisol-secreting adenoma	−	+	Long/Short	−	+	−	+
14	Cortisol-secreting adenoma	+	+	Long/Short	−	+	−	+
15	Nonfunctioning adenoma	−	+	Long/Short	−	+	−	+
16	Nonfunctioning adenoma	−	+	Long/Short	−	+	−	+
17	Aldosterone-secreting carcinoma	−	−	−	−	−	−	+
18	Aldosterone-secreting carcinoma	−	−	−	−	−	−	+
19	Cortisol-secreting carcinoma	−	+	Long	−	+	−	+
20	Cortisol-secreting carcinoma	−	+	Long	−	+	−	+
21	Androgen-secreting carcinoma	−	−	−	−	−	−	+
22	Androgen-secreting carcinoma	−	−	−	−	−	−	+
23	Pheochromocytoma	−	+	Long/Short	−	+	+	+
24	Pheochromocytoma	−	+	Long/Short	−	+	+	+
25	Pheochromocytoma	−	+	Long/Short	−	+	+	+
Control	Human brain	+	+	Long/Short	+	+	+	+

mocriptine or cabergoline administration, the cabergoline-induced however being significantly higher than the bromocriptine-induced inhibition (Fig. 5).

Discussion

The results of the current study demonstrated that: 1) D_1-like and D_2-like receptors are both expressed in the normal adrenal gland; 2) D_2 receptors are expressed in cells of all areas of the normal adrenal gland, although they are mainly localized in the zona glomerulosa and zona reticularis of the adrenal cortex and in the adrenal medulla and scantly in the zona fasciculata of the adrenal cortex; 3) D_2-like receptors are expressed in different categories of adrenal benign tumors, such as aldosterone- and cortisol-secreting and clinically nonfunctioning adenomas and in malignant tumors, such as cortisol-secreting carcinomas, as well as in pheochromocytomas; 4) a high concentration of the dopamine agonist cabergoline, but not bromocriptine, significantly inhibits both baseline and ACTH-stimulated aldosterone, but not cortisol and androstenedione secretion; and 5) cabergoline is more effective than bromocriptine in inhibiting angiotensin-II stimulated aldosterone secretion.

DR expression in the adrenal gland has been first hypothesized after the evidence for a role of dopamine in the control of aldosterone secretion derived from *in vivo* studies in experimental animals as well as in humans. Indeed, the administration of the D_2 antagonist metoclopramide to both rats and humans was demonstrated to increase plasma aldosterone levels without influencing any stimulator of aldosterone release, an effect blocked by the iv injection of dopamine (8–10). However, the administration of dopamine or the dopamine agonist bromocriptine did not modify plasma aldosterone levels (11). These observations suggested that aldosterone production is under maximum tonic dopaminergic inhibition. Subsequent studies demonstrated that the sodium balance state is crucial for the effect of dopamine or dopamine agonists on aldosterone secretion. Indeed, dopamine and D_2 receptor agonists were shown to inhibit angiotensin II-stimulated and upright posture-induced increased aldosterone secretion in sodium-depleted, and not in sodium-repleted, normal subjects (18, 19). On the other hand, *in vitro* studies with isolated adrenal glomerulosa cells demonstrated that the activation of D_2 receptors resulted in a remarkable inhibition of angiotensin II-induced aldosterone secretion, whereas it did not influence basal and ACTH-induced aldosterone secretion (13). These studies suggested D_2- or D_2-like receptor expression in cells of the zona glomerulosa of the adrenal cortex and a selective functional interaction between dopamine and angiotensin II in the regulation of aldosterone secretion. No study has definitely

TABLE 3. DR expression in normal adrenal, adrenal hyperplasia, and cortisol-secreting adrenal adenoma and carcinoma determined by Scatchard analysis of ^{125}I-epidepride binding on membrane homogenates

Cases	^{125}I-epidepride binding	
	Bmax (fmol/mg protein)	Kd (nM)
Normal adrenal (no. 1)	25 ± 0.9	0.21 ± 0.03
Adrenal hyperplasia (no. 4)	36 ± 0.7	0.19 ± 0.02
Cortisol-secreting adrenal adenoma (no. 9)	40 ± 0.8	0.22 ± 0.05
Cortisol-secreting adrenal carcinoma (no. 14)	22 ± 0.5	0.24 ± 0.07
Rat brain basal ganglia	80 ± 12.5	0.18 ± 0.01

The data represent the mean ± SE of three different experiments for each case.

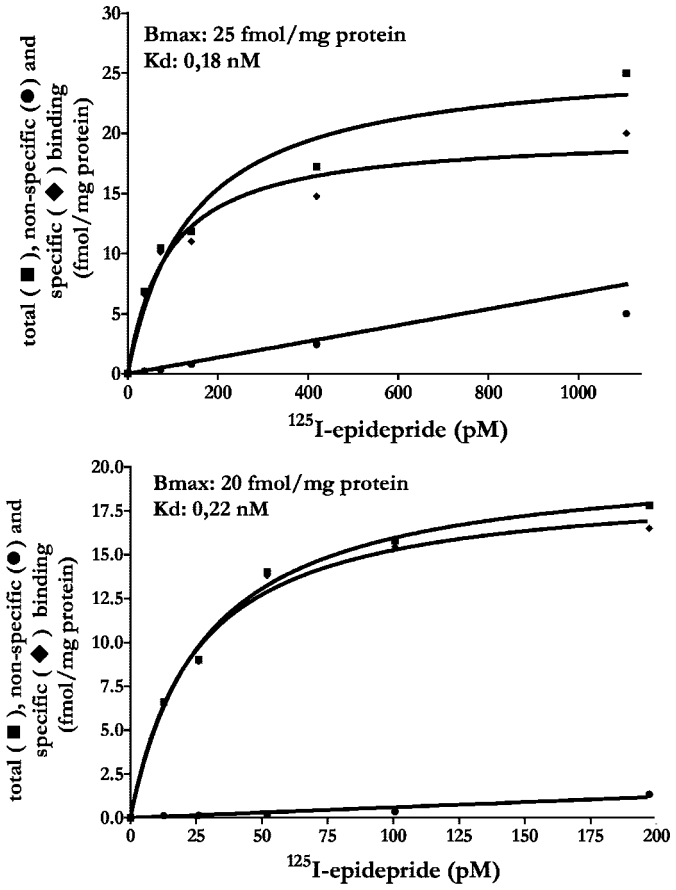

FIG. 1. Representation of the binding of ^{125}I-epidepride binding to membrane homogenate preparation of a normal adrenal (*top*) and a cortisol-producing adrenal adenoma (*bottom*). ■, Total binding; ●, nonspecific binding in presence of 1 μM cabergoline; ◆, specific binding (total minus nonspecific binding).

evaluated the possible effect of dopamine or dopamine agonists on cortisol or androgen secretion by the adrenal gland.

DR expression in the adrenal gland has been subsequently confirmed by RLBS, demonstrating the presence of specific and saturable binding sites for radiolabeled dopamine antagonists such as spiperone, which binds both D_1-like and D_2-like receptors, and sulpiride, which selectively binds D_2-like receptors in the adrenal cortex of experimental animals as well as humans (2, 12). These studies demonstrated that both D_1-like and D_2-like receptors are expressed in the human adrenal cortex. On the other hand, the expression of D_1-like and D_2-like receptors in the adrenal medulla has been clearly demonstrated to mediate the dopamine regulation of catecholamine secretion (5–7). However, no study has evaluated the DR subtypes and D_2 isoforms expressed in the various areas of adrenal cortex.

The presence of D_1 receptor has been demonstrated in the zona glomerulosa of the rat adrenal gland by IHC and ISH studies, suggesting a possible role of this receptor in regulating the dopamine effects on aldosterone secretion (20). Moreover, a recent study evaluated the expression of D_2-like receptor subtypes in normal adrenals by RT-PCR and ISH studies, demonstrating that D_2 and D_4 receptors are both expressed in the adrenal medulla and in all three areas of the adrenal cortex (14). The same study demonstrated that D_4 is the predominant D_2-like receptor expressed in the adrenal cortex and that the zona glomerulosa is the area with higher density of D_2-like receptors (14). The results of the current study are in line with these latter ones, as demonstrated by the expression of both D_2 and D_4 receptors in the normal adrenal gland. In addition, the current study reported, for the first time, that both D_1 and D_5 receptors are expressed in the adrenal gland. Therefore, four of the five different DR are expressed in the human normal adrenal gland, suggesting the existence of a complex regulation of adrenal function by dopamine in physiological conditions. As far as the localization is concerned, the heterogeneous D_2 expression in the different areas, as was demonstrated by the level of D_2 gene product expression by ISH in a previous study (14), is confirmed in the present study at level of D_2 protein expression by IHC. Indeed, the expression of this DR subtype in all adrenal areas, but mainly in the zona glomerulosa and reticularis and, to a lesser extent, in the zona fasciculata and adrenal medulla, is clearly demonstrated. These findings suggested a possible role for dopamine in the regulation of the secretion of all different adrenal hormones. However, neither bromocriptine nor cabergoline, two different dopamine agonists, is able to induce a significant *in vitro* effect on both basal and ACTH-stimulated cortisol and androstenedione secretion. As far as aldosterone is concerned, as previously described (13), bromocriptine did not induce an effect on the secretion of this hormone, except a slight nonsignificant increase at low dose and a slight nonsignificant decrease of hormone secretion at high dose of the drugs, a secretion pattern which was also observed for cortisol and androstenedione secretion. However, cabergoline induced a significant stimulation at low dose and a significant inhibition at high dose of baseline and ACTH-stimulated aldosterone secretion. On the other hand, both bromocriptine and cabergoline induced a significant inhibition of the angiotensin II-induced aldosterone secretion, cabergoline being significantly more effective than bromocriptine. These differential effects of the two dopamine agonists may be explained by the different pharmacological characteristics of bro-

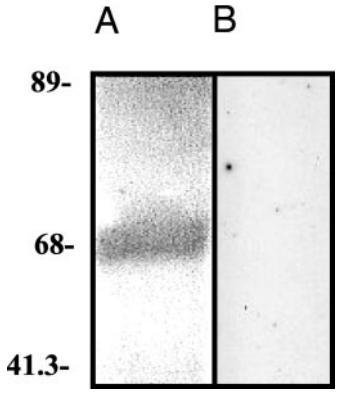

FIG. 2. Immunoblot of glycoproteins derived from a human pheochromocytoma with D_2 receptor antibody. The figure shows the results without (A) and with (B) the preincubation of the antibody with the antigen. The specificity of the immunoblot is demonstrated by the complete disappearance of the signal after the preincubation of the antibody with the antigen (D_2 receptor peptide).

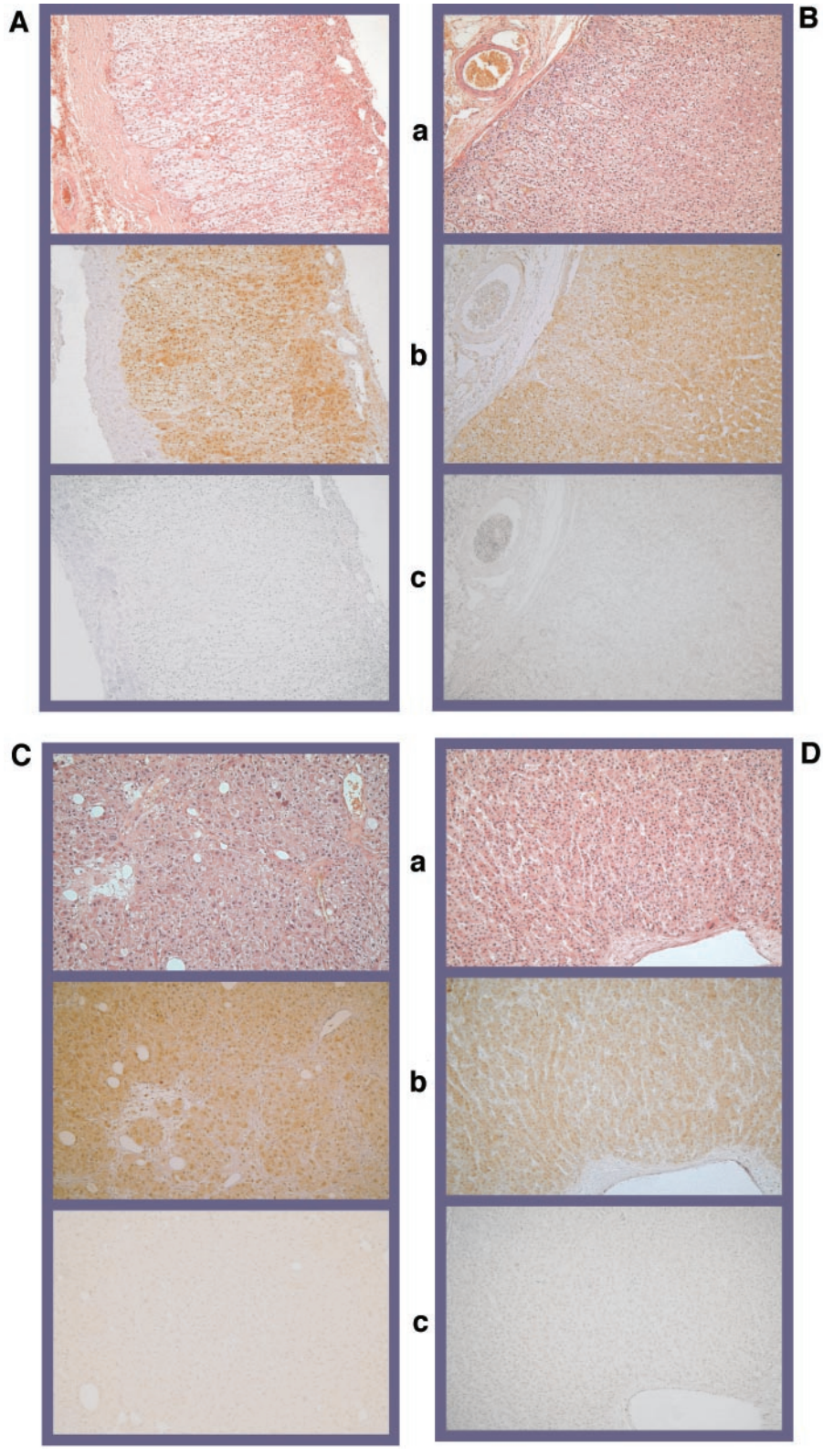

FIG. 3. Expression of D_2 receptor by IHC in one case of normal adrenal (A), one case of adrenal hyperplasia (B), one case of cortisol-producing adrenal adenoma (C), and one case of adrenal carcinoma (D). The IHC study has been performed on formalin-fixed and paraffin-embedded sections of the tumors. a, Hematoxylin-eosin stained section; b, D_2 receptor immunostaining performed with a specific polyclonal D_2 receptor antibody; c, D_2 receptor immunostaining after the preincubation of the antibody with the specific antigen. A strong specific D_2 immunostaining is evident in the zona glomerulosa and reticularis of normal and hyperplastic adrenal as well as in the tumoral cells of cortisol-producing adenoma and carcinomas, whereas a faint, but specific, immunostaining is evident in the zona fasciculata of normal and hyperplastic adrenals. The specificity of the immunostaining is demonstrated by the disappearance of immunostaining after the preabsorption of the antibody with the specific antigen (D_2 receptor peptide). Magnification, ×100.

mocriptine and cabergoline, which displays a higher affinity for D_2-like receptors (21, 22). Moreover, because D_4 seems to play a role in the aldosterone secretion, a different effect of bromocriptine and cabergoline via D_4 receptors cannot be ruled out. The lack of a clear-cut *in vitro* effect of dopamine agonists on hormone secretion in normal adrenal may be related also to opposite dopamine agonist effects mediated by two different DR expressed in the same cells. Indeed,

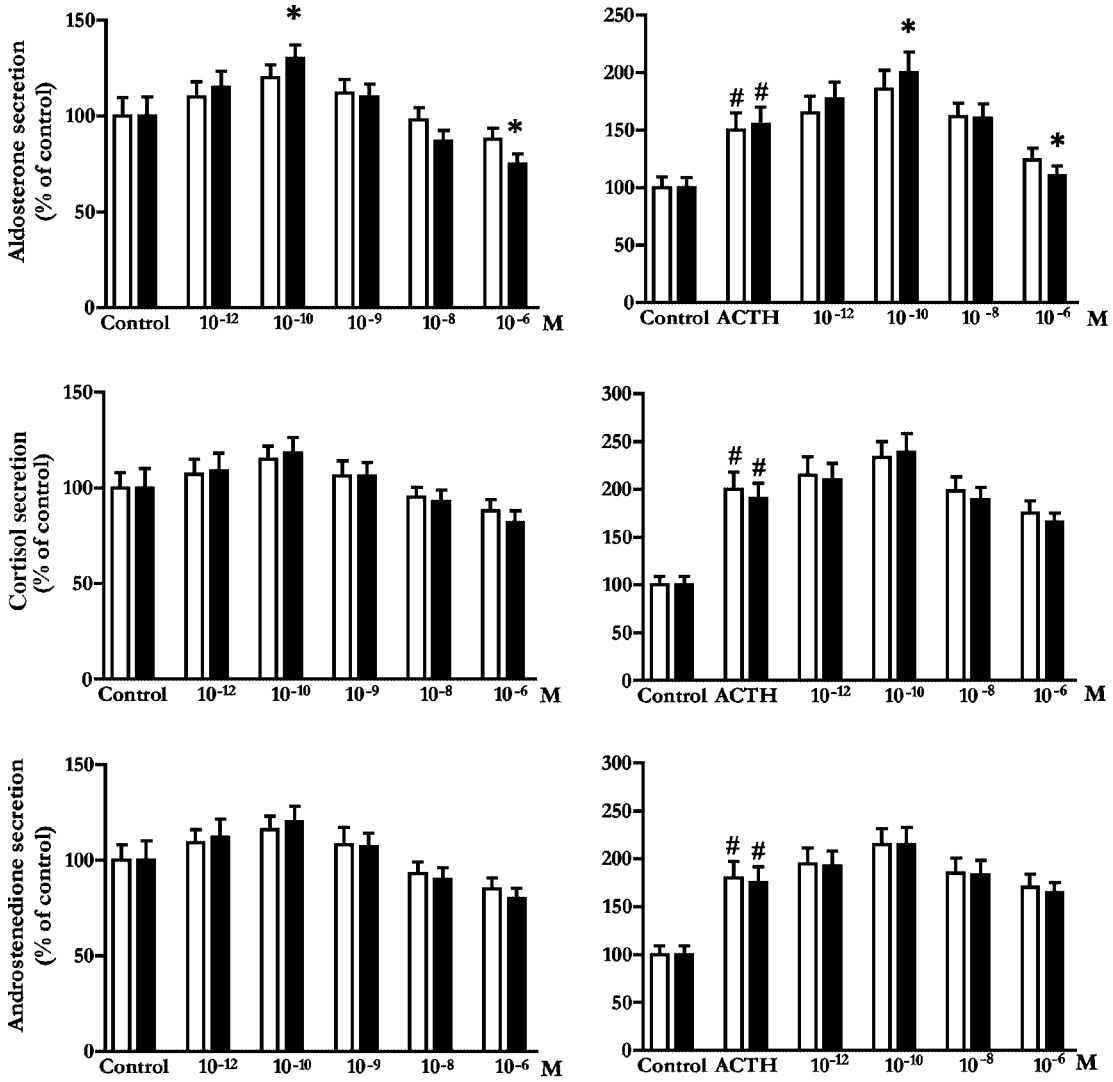

FIG. 4. Effect of *in vitro* administration of bromocriptine (□) and cabergoline (■) on baseline (*left*) and ACTH-stimulated (*right*) aldosterone (*top*), cortisol (*middle*), and androstenedione (*bottom*) secretion in cell cultures derived by three different adrenal hyperplasia. Values are expressed as secretion percentage and are mean ± SE (n = 3 treatment; n = 4 per treatment). *, $P < 0.05$ compared with control in baseline studies and to ACTH challenge in stimulated studies; #, $P < 0.05$ compared with control in ACTH-stimulated studies).

opposite effects of dopamine antagonists on aldosterone secretion have been postulated to be mediated by D_2 and D_4 in a previous study (14). However, on the basis of the results of the present study, opposite effects of dopamine agonists mediated by a D_1-like and a D_2-like receptor may be also hypothesized to explain the minor effects exerted by bromocriptine and cabergoline on hormone secretion in normal adrenal. It must, however, be outlined that the results of the present study seem to confirm the concept that aldosterone production is under maximum tonic dopaminergic inhibition in normal conditions.

DR receptor expression in adrenal tumors has been poorly investigated. Indeed, D_2-like receptors have been found to be expressed in pheochromocytomas and in aldosterone-secreting adrenal adenomas (6, 14). The current study is the first one evaluating DR expression in a wide series of various benign and malignant adrenal tumors. The expression of different DR expression has been found in some categories of both benign and malignant adrenal tumors. In particular, aldosterone-secreting adrenal adenomas and pheochromocytomas express both D_2 and D_4 receptors and may also express D_5 receptors, suggesting that this latter receptor may be constitutively expressed in the zona glomerulosa as well as in the medulla of the normal adrenal gland and therefore play a physiological role in aldosterone and catecholamine secretion together with the D_2-like receptors. Moreover, both cortisol-secreting adrenal adenomas and carcinomas express both D_2 and D_4 receptors, whereas D_1 receptors are also expressed in part of the adenomas. This suggests that D_1 may be constitutively expressed in the zona fasciculata of the normal adrenal gland, where it may play a physiological role in cortisol secretion together with the D_2-like receptors. In addition, clinically nonfunctioning adrenal adenomas express D_2 and D_4 receptors. However, neither aldosterone-secreting nor androgen-secreting adrenal carcinomas seem to express any DR in the series evaluated in the present study.

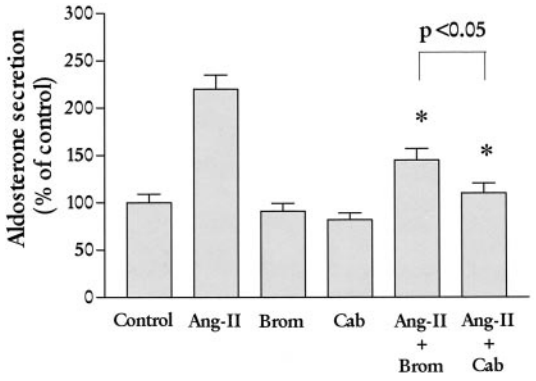

FIG. 5. Effect of *in vitro* administration of bromocriptine (Brom) and cabergoline (Cab) on baseline and angiotensin (Ang)-II-stimulated aldosterone secretion in cell cultures derived by two different adrenal hyperplasia. Values are expressed as secretion percentage and are mean ± SE (n = 2 treatment; n = 4 per treatment). *, $P < 0.05$ compared with angiotensin-II stimulated aldosterone secretion.

Anyhow, DR, in particular D_2-like receptor expression, which is usually associated with inhibitory effects, raises the hypothesis of a possible effect of dopamine agonists in the control of hormonal hypersecretion associated with some adrenal tumors.

The last finding of the current study is represented by the evidence of the expression of both D_2 receptor isoforms (D_{2short} and D_{2long}), in all D_2-positive adrenal samples, with the exception of one aldosterone-secreting adenoma and the cortisol-secreting carcinomas, which express only D_{2long} isoform. The D_2 isoforms derive from an alternative splicing of the D_2 receptor gene product (23). The affinity of dopamine and dopamine agonists for D_2 receptor isoforms is nearly identical, but the intracellular signaling pathways activated by the binding of the receptor with the ligand seem to be different for each isoform. Therefore, heterogeneous G protein coupling would allow a variable second-messenger activation and, probably, different effects by the two D_2 receptor isoforms (24, 25). In particular, the role of D_2 receptor isoforms has been recently studied in the different pituitary tumors. Indeed, D_{2short} isoform has been suggested to be associated with a more potent dopaminergic effect, compared with D_{2long} isoform, both in clinically nonfunctioning (26, 27) and ACTH-secreting pituitary tumors (28). A possible major role of D_{2short}, rather than D_{2long}, isoform in the control of hormone secretion in normal adrenal and adrenal tumors as well cannot be ruled out, although this likely has some significance only or mainly in cortisol-secreting carcinomas, which is the only adrenal tumor expressing one isoform of the D_2 receptors.

In conclusion, the current study demonstrated that both D_1-like (namely D_1 and D_5) and D_2-like (namely D_2 and D_4) receptors are expressed in the normal adrenal gland and may be expressed in adrenal adenomas or carcinomas. Although bromocriptine and cabergoline are able to induce only a minor inhibition of adrenal hormones secretion in nontumoral adrenal gland *in vitro*, a potential use of dopamine agonists *in vivo* in the treatment of adrenal tumors cannot be excluded.

Acknowledgments

We are greatly indebted to Pharmacia-Pfizer for its technical support of the study.

Received October 6, 2003. Accepted May 10, 2004.

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This work was supported by Pharmacia-Pfizer and in part by a grant from the Italian Ministry of University and Research (no. 2003-068735).

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