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Pharmacological and Molecular Characterization of 5-Hydroxytryptamine$_7$ Receptors in the Rat Adrenal Gland

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ABSTRACT

Serotonin (5-hydroxytryptamine; 5-HT) is a potent stimulator of aldosterone secretion in the rat adrenal gland but the type of receptor involved in the mechanism of action of 5-HT remains unknown. The aim of the present study was to determine the pharmacological profile and to clone the receptor responsible for the corticotropic effect of 5-HT in rat glomerulosa cells. A series of 10 serotonergic receptor agonists and 12 receptor antagonists was used to characterize the receptor mediating the effect of 5-HT on aldosterone secretion from perifused rat adrenocortical slices. Correlation analysis between the potencies of the different compounds in our model and those previously reported for various recombinant 5-HT receptors showed that the rat adrenal 5-HT receptor exhibits the same pharmacological profile as the 5-HT$_7$ receptor transiently expressed in COS-7 cells ($r = 0.82$ for agonists, $p < .05$; $r = 0.83$ for antagonists, $p < .01$). Polymerase chain reaction with specific primers revealed the expression of 5-HT$_7$ receptor mRNA in the rat adrenal gland. Cloning of the polymerase chain reaction product confirmed that the amplified DNA corresponded to the 5-HT$_7$ receptor cDNA sequence. Western blot analysis showed the presence of a protein with an apparent molecular mass of 66 kDa in the adrenal cortex but not in the medulla. Taken together, these data demonstrate that the rat adrenal glomerulosa expresses functional 5-HT$_7$ receptors. Rat glomerulosa cells will thus provide a robust and sensitive bioassay for future studies on native 5-HT$_7$ receptors.

The various effects of serotonin (5-hydroxytryptamine; 5-HT) on the central nervous system and peripheral organs are mediated through activation of multiple types of receptors (Hoyer and Martin, 1997). Most of the 5-HT receptor subtypes currently known have been initially identified by pharmacological approaches and subsequently cloned, i.e., 5-HT$_{1A,\ 1B/D}$, 5-HT$_{2A,\ 2C}$, 5-HT$_3$, and 5-HT$_4$ (Peroutka, 1990) whereas others, i.e., 5-ht$_5$, 5-ht$_6$, and 5-HT$_7$ receptors, have been directly characterized by molecular cloning (Boess and Martin, 1994; Branchek and Zgombick, 1997). Heterologous expression of recombinant receptors in cell lines has been widely used to determine the pharmacological profiles and transduction pathways of the cloned receptors. However, several reports have revealed unusual drug-receptor interaction behaviors depending on the densities of receptors transfected and/or G protein present in the different cell lines (Kenakin, 1997, for review). Thus, physiological models are badly needed for characterizing the functional properties of newly identified 5-HT receptor types, for which little or no functional data are available.

It is now well established that 5-HT is a potent stimulator of aldosterone secretion (Lefebvre et al., 1998, for review). In human and frog, the effect of 5-HT on adrenocortical cells is clearly mediated through a 5-HT$_4$ receptor subtype (Idres et al., 1991; Lefebvre et al., 1992, 1993; Contesse et al., 1994). In contrast, controversial data have been reported concerning the type of receptor mediating the corticotropic effect of 5-HT on rat glomerulosa cells. Early studies have shown that 5-HT stimulates aldosterone secretion by the rat adrenal gland (Müller and Ziegler, 1968; Haning et al., 1970) and it

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ABBREVIATIONS: 5-CT, 5-carboxamidotryptamine; 5-HT, 5-hydroxytryptamine; 5-MeOT, 5-methoxytryptamine; 5-MeODMT, *N*, *N*-dimethyl-5-methoxytryptamine; 8-OH-DPAT, (±)-8-hydroxy-2-(di-*n*-propylamino)tetralin; DOI, (±)-1-(2,5-dimethoxy-4-iodophenyl)-2 aminopropane (*R*, *S*)-zacopride, (*R*, *S*)-4-amino-*N*-(1-azabicyclo-[2.2.2]oct-3-yl)-5-chloro-2-methoxybenzamide; BIMU 8, endo-*N*-(8-methyl-8-azabicyclo-[3.2.1]oct-3-yl)-2,3-dihydro-(1-methyl)ethyl-2-oxo-1*H*-benzimidazolone-1-carboxamide; GR 113808, [1-[2-(methylsulfonylamino)ethyl]-4-piperidinyl]methyl 1-methyl-1*H*-indole 3-carboxylate; DAU 6285, endo-8-methyl-8-azabicyclo[3.2.1]oct-3-yl)-2,3-dihydro-6-methoxy-2-oxo-1*H*-benzimidazolone-1-carboxylate; RT-PCR, reverse transcription-polymerase chain reaction; HBS, Hanks' buffered saline; DMSO, dimethyl sulfoxide; GAPDH, glyceraldehyde-3-phosphate dehyrogenase.

has been subsequently demonstrated that the effect of 5-HT is associated with activation of adenylyl cyclase (Williams et al., 1984; Matsuoka et al., 1985). Because the action of 5-HT on both aldosterone production and cAMP formation is inhibited by ketanserin (Williams et al., 1984; Matsuoka et al., 1985; Rocco et al., 1986), it has been purported that in rat glomerulosa cells 5-HT stimulates a 5-HT$_2$ receptor positively coupled to adenylyl cyclase. However, it is now firmly established that stimulation of 5-HT$_2$ receptors causes selective activation of phospholipase C and does not affect cAMP formation (Conn et al., 1986). Because, in rat glomerulosa cells, 5-HT is totally devoid of action on phospholipid hydrolysis (Rocco et al., 1990), it clearly appears that the effect of 5-HT on aldosterone secretion cannot be mediated through activation of 5-HT$_2$ receptors (Lefebvre et al., 1998).

In the present study, we have investigated the effects of a series of 10 serotonergic receptor agonists and 12 receptor antagonists to determine the pharmacological profile of the receptor that mediates the effect of 5-HT on aldosterone secretion in the rat adrenal gland. Because the data suggested the involvement of a 5-HT$_7$ receptor subtype in the corticotropic activity of 5-HT, we have subsequently searched for the occurrence of 5-HT$_7$ receptor mRNA by reverse transcription-polymerase chain reaction (RT-PCR) amplification and molecular cloning, and we have investigated the presence of the receptor protein by Western blot analysis.

Materials and Methods

Animals and Tissue Preparation. Male Wistar rats weighing 250 to 350 g were maintained under controlled conditions of temperature (22°) under an established photoperiod (lights on from 7:00 AM to 7:00 PM). Rats had free access to laboratory chow (UAR, Epinay-sur-Orge, France) and water. All manipulations were performed according to the recommendations of the French ethical committee and under the supervision of authorized investigators. The animals were sacrificed by decapitation between 8:30 and 9:30 AM. The adrenal glands were quickly removed and dissected free of adherent fat. The cortex, separated from the medulla, was sliced and preincubated in Hanks' buffered saline (HBS) solution (130 mM NaCl, 3.5 mM KCl, 1.8 mM $CaCl_2$, 0.5 mM $MgCl_2$, 2.5 mM $NaHCO_3$, 5 mM HEPES, supplemented with 1 g/liter BSA, 1 g/liter glucose, and 1% of the antimycotic/antibiotic solution). The HBS solution was gassed with a 95% O_2/5% CO_2 mixture, and the pH was adjusted at 7.35.

Reagents. Initial solutions were made in HBS or dimethyl sulfoxide (DMSO), depending on the solubility of each compound. The final concentration of DMSO was 0.01%. Tryptamine, 5-HT, 5-methoxytryptamine (5-MeOT), pimozide, clozapine, mianserin, and cyproheptadine were purchased from Sigma (St. Louis, MO). Pergolide, 5-carboxamidotryptamine (5-CT), *N,N*-dimethyl-5-methoxytryptamine (5-MeODMT), (±)-8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT), (±)-1-(2,5-dimethoxy-4-iodophenyl)-2 aminopropane (DOI), methiothepin, *R*(+)lisuride, metergoline, and 1-(1-naphtyl)piperazine were obtained from RBI (Bioblock Scientific, Illkirch, France). Mesulergine was provided by Sandoz (Basel, Switzerland). Ketanserin was supplied by Janssen Research Foundation (Beerse, Belgium). Endo-*N*-(8-methyl-8-azabicyclo-[3.2.1]oct-3-yl)-2,3-dihydro-(1-methyl)ethyl-2-oxo-1*H*-benzimidazolone-1-carboxamide (BIMU 8) and endo-8-methyl-8-azabicyclo[3.2.1]oct-3-yl)-2,3-dihydro-6-methoxy-2-oxo-1*H*-benzimidazolone-1-carboxylate (DAU 6285) were generous gifts from Boehringer Ingelheim (Milan, Italy). [1-[2-(methylsulfonylamino)ethyl]-4-piperidinyl]methyl 1-methyl-1*H*-indole 3-carboxylate (GR 113808) was a gift from Glaxo (Greenford, UK). (*R*, *S*)-4-Amino-*N*-(1-azabicyclo-[2.2.2]oct-3-yl)-5-chloro-2-methoxybenzamide [(*R*, *S*)-zacopride] was kindly provided by Synthélabo (Rueil-Malmaison, France). The polyclonal antibody raised in rabbit against the 5-HT$_7$ receptor was obtained from Diasorin (Stillwater, MN). [1,2,6,7-^{3}H]Aldosterone was purchased from Amersham International (Les Ulis, France).

Perifusion Experiments. The effect of test substances on aldosterone secretion was studied by means of a perifusion technique, as described previously (Feuilloley et al., 1986). Briefly, slices of rat adrenal cortex were rinsed twice with fresh medium and layered between several beds of Bio-Gel P2 (Bio-Rad Laboratories, Richmond, CA) into perifusion chambers (equivalent of 2 adrenal glands/chamber). The adrenal tissue was continuously perifused with gassed HBS solution at a constant flow rate (200 μl/min) and temperature (37°). The glands were allowed to stabilize for 5 h before any test substance was added to reach a steady-state level of aldosterone secretion. After stabilization, the mean secretion rate of aldosterone in basal conditions was 194 ± 44 pg/min/adrenal. Secretagogues were dissolved in gassed HBS solution immediately before use and infused into the columns at the same flow rate as the HBS solution alone, by means of a multichannel peristaltic pump (Desaga, Heidelberg, Germany). Several antagonists were initially dissolved in DMSO so that the final concentration of DMSO in the perifusion medium was 0.01%. At this concentration, DMSO had no effect on spontaneous or 5-HT-induced aldosterone secretion. Fractions of effluent perifusate were collected every 5 min (1 ml/fraction), and the tubes were immediately frozen until the aldosterone assay.

Aldosterone concentration was determined by radioimmunoassay, without prior extraction, in 100- to 200-μl aliquots from each fraction of perifusate. The specificity characteristics of the radioimmunoassay have been reported previously (Leboulenger et al., 1982). The assay was sensitive enough to detect 5 pg of aldosterone. The intra-assay and interassay coefficients of variation were lower than 4 and 10%, respectively. The recovery of the assay was 95.2 ± 4.4%.

Each perifusion pattern was established as the mean profile of aldosterone secretion (± S.E.M.) calculated from at least three independent experiments. The levels of aldosterone released were expressed as change in picograms per minute per adrenal. The concentration-response curves obtained with serotonin and serotonergic agonists were fitted using the SigmaPlot program (Jandel Scientific, San Rafael, CA), and E_{max} (maximum response) and pEC_{50} values (negative logarithm of EC_{50}, the agonist concentration producing 50% of the maximum aldosterone secretion) were derived from this analysis. To determine the affinities of receptor antagonists, the concentration-response curves for 5-HT were performed in the absence or presence of the antagonist. Apparent antagonist dissociation constants (K_B) were determined for each antagonist according to the following equation: K_B = [B]/(dose ratio − 1), where [B] is the concentration of the antagonist, and the dose ratio is the quotient of the EC_{50} of the agonist in the presence of the antagonist to the EC_{50} of the agonist in the absence of antagonist. The results were then expressed as the negative logarithm of K_B ($-\log K_B = pK_B$) (Furchgott, 1972). At least three independent experiments were performed for each concentration-response curve.

RT-PCR. The adrenal glands were collected, the zona glomerulosa and zona fasciculata/reticularis were carefully dissected, and the tissues were immediately frozen on dry ice. Total RNA was extracted using a single-step procedure according to Chomczynski and Sacchi (1987) using the Tri reagent (Sigma). The concentration and purity of RNA were determined by spectrophotometry analysis (UV-1605; Shimadzu, Kyoto, Japan). The RT reaction was carried out in a total volume of 20 μl in the presence of 5 μg of total RNA, 0.5 μg oligo(dT)15 primer, and 200 U Maloney murine leukemia virus reverse transcriptase, in a buffer containing 50 mM Tris-HCl, 75 mM KCl, 3 mM $MgCl_2$, and 10 mM dithiothreitol. The reaction mixture was incubated at 42° for 1 h.

Two primers were designed from the cloned rat 5-HT$_7$ receptor sequence (Lovenberg et al., 1993; accession no. L22558) as follows: 5′-CTGACGATCGCAGGCAACTGCCT-3′ and 5′-TGCCTGCAGCAGAGAGCTTCCGGT-3′ corresponding to bases 404 to 426 and 1344

to 1367, respectively, of the rat 5-HT_7 receptor sequence. Two other primers (5′-TGCTGAGTAYGTCGTGGAGTC-3′ and 5′-TTGGTGGTGCAGGAKGCATTGC-3′), corresponding to bases 297 to 317 and 467 to 488, respectively, of the cloned glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sequence (Tso et al., 1985; accession no. M17701), were used in control experiments.

PCR was carried out in a reaction volume of 50 μl containing 1/10 RT reaction and primers (10 pmol) in 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 2.5 mM $MgCl_2$, 0.1% Triton X-100, 0.2 mM each dNTP, and 2.5 U *Taq* polymerase. After a 3-min denaturation at 94°, 30 cycles of amplification were performed (30-s denaturation at 94°, 1-min annealing at 48°, and 1-min extension at 72°), in a DNA Thermal Cycler (Perkin Elmer, Courtaboeuf, France). The PCR products were separated by electrophoresis in a 2% agarose gel and visualized by UV illumination in the presence of ethidium bromide. Individual bands were excised, purified using the Geneclean I kit (BIO 101, Inc., Vista, CA), and ligated into the pGEM-T vector (Promega). Each band was characterized by restriction mapping and subsequently sequenced using the Thermosequencage Kit (Amersham) on a Li-Cor 4000L DNA Sequencer (Science Tec, Les Ulis, France).

Tissue Extraction and Western Blot Analysis. Twenty rat adrenal glands were collected and the glomerulosa, fasciculata/reticularis, and medullary zones were carefully dissected. The tissues were homogenized in 10 mM Tris-HCl, pH 7.4, containing 0.05% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride. The extracts were centrifuged at 12,000*g* for 10 min at 4°, and the pellets were suspended in 60 mM Tris-HCl, pH 6.8, containing 10% glycerol, 0.001% (w/v) bromophenol blue and 3% (w/v) SDS. The proteins were solubilized for 3 h at room temperature. The mixture was centrifuged at 12,000*g* for 10 min at 4° and the supernatant was applied to a 10% polyacrylamide gel with a 5% stacking gel. Proteins were electroblotted onto a nitrocellulose sheet (Amersham Pharmacia Biotech) and revealed with the antibody against the 5-HT_7 receptor, using a chemiluminescence detection kit (Amersham Pharmacia Biotech).

Results

Effect of 5-HT and Serotonergic Agonists on Aldosterone Secretion. Administration of graded concentrations of 5-HT (1 nM to 10 μM) during 30 min to perifused rat adrenal slices induced a concentration-dependent increase in aldosterone secretion (Fig. 1A). The minimum effective concentration was 10 nM, and maximum stimulation was observed at a concentration of 0.3 μM. A representative concentration-response curve is shown in Fig. 1B. The concentration of 5-HT that produced half-maximum response (EC_{50}) was 30 nM in this experiment (mean pEC_{50} value of 7.20 ± 0.13 and E_{max} of 707.6 ± 38.9 Δpg/min/adrenal; 17 experiments). Hill plot analysis of the concentration-response data (Fig. 1B, inset) revealed that the activation of aldosterone secretion occurred with a first-order kinetics, indicating that 5-HT interacted with a single population of receptors (n_H = 0.943). Two series of control experiments showed that long-term exposure of rat adrenal slices to 5-HT did not cause desensitization of the serotonergic receptor: 1) the magnitude of the stimulation of aldosterone secretion induced by a single administration of 5-HT (1 μM; 30 min) to perifused rat adrenocortical slices (776 ± 57 Δpg/min/adrenal; four experiments) was not significantly different from the maximum response (E_{max}) observed during administration of cumulative concentrations of 5-HT (Table 1); and 2) the amplitude and the kinetics of the responses monitored during prolonged exposure (4 h) to 1 μM 5-HT or to 8 mM KCl were virtually identical (data not shown).

A series of 10 serotonergic agonists were tested for their ability to stimulate aldosterone secretion from perifused rat adrenocortical slices. Graded concentrations of six agonists caused smooth monophasic response curves (n_H ~1.0) (Fig. 2). The potency and efficacy of the various compounds are summarized in Table 1. All the indolealkylamines tested displayed agonistic activity, 5-CT being by far the most potent compound in stimulating aldosterone secretion. For example, 5-CT was 10 times more potent than 5-HT or the ergoline derivative pergolide. In addition, 5-CT was >300 times more potent than the tryptamine derivatives 5-MeOT or 5-MeODMT, and >1500 times more potent than tryptamine. The aminotetralin derivative 8-OH-DPAT, a full 5-HT_{1A} receptor agonist, was 50 times less potent than 5-HT (Table 1). All of these agonists were capable of stimulating aldosterone secretion to a level ranging from 62 to 100% as compared with 5-HT (Fig. 2 and Table 1). The arylpiperazine derivative 1-(1-naphthyl)piperazine, a 5-HT_1 receptor agonist and a 5-HT_2 receptor antagonist, was a weak partial agonist (pEC_{50} = 6.48 ± 0.17; E_{max} = 80.4 ± 12.6 Δpg/min/adrenal; four experiments; data not shown). Finally, DOI, a potent and selective 5-HT_2 receptor agonist, as well as the

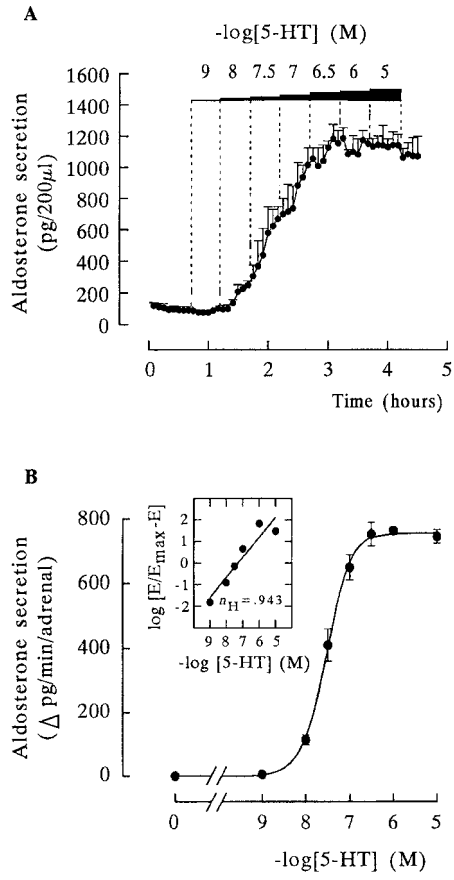

Fig. 1. Effect of graded concentrations of 5-HT on aldosterone secretion by perifused rat adrenocortical slices. A, typical perifusion profile illustrating the effects of increasing concentrations of 1 nM to 10 μM 5-HT on aldosterone secretion. B, semilogarithmic plot showing the effect of graded concentrations of 5-HT on the amplitude of aldosterone secretion. Experimental values were calculated from data similar to those presented in Fig. 1A. The net increase in aldosterone secretion (Δpg/min/adrenal), measured in the three consecutive fractions collected just after each pulse of 5-HT, was plotted against 5-HT concentration. Data were analyzed by computer-assisted nonlinear regression analysis (SigmaPlot, Jandel Scientific) Inset, Hill plot of the concentration-response curve.

benzamide derivative (*R*,*S*)-zacopride and the benzimidazolone derivative BIMU 8, two selective 5-HT$_4$ receptor agonists, had no effect on aldosterone secretion even at a concentration of 100 μM (Fig. 2; Table 1).

To verify that 5-HT and the 5-HT agonists actually interacted with the same receptor to stimulate aldosterone secretion, the effects of concomitant administration of 5-HT and 5-HT agonists were examined (Fig. 3). The magnitude of the stimulation of aldosterone secretion induced by a saturating concentration of 5-HT (1 μM) was not enhanced by addition of maximum effective doses of 5-CT (1 μM), 5-MeOT, 5-MeODMT, or 8-OH-DPAT (10 μM each). As a control, the stimulatory effect of 10 mM KCl on aldosterone secretion was found to be significantly higher (1103 ± 136 Δpg/min/adrenal; three experiments) than that of 1 μM 5-HT (680 ± 142 Δpg/min/adrenal; four experiments), indicating that the secretory activity of adrenocortical cells was not maximally stimulated by 1 μM 5-HT (Fig. 3).

Effects of Serotonergic Antagonists. Various compounds were tested for their ability to inhibit 5-HT-induced aldosterone secretion from perifused rat adrenocortical slices. The average pK_B values for compounds exhibiting antagonistic activity are shown in Table 2. At the concentrations used, none of the antagonists tested induced any modification of basal aldosterone secretion (data not shown). The most potent inhibitor of 5-HT-evoked aldosterone secretion was methiothepin (pK_B = 10.13 ± 0.26), a nonselective 5-HT$_1$/5-HT$_2$ receptor antagonist (Fig. 4A; Table 2). The ergoline derivatives *R*(+)lisuride, metergoline, and mesulergine were also highly potent antagonists (Fig. 4B; Table 2). The fact that the pK_B values of metergoline and mesulergine were lower than that of *R*(+)lisuride (Table 2) indicates that methyl substitution on the indole nitrogen decreases the potency compared with hydrogen substitution. Two antipsychotic agents were also tested for their ability to inhibit 5-HT-induced aldosterone secretion: the diphenylbutylpiperidine derivative pimozide exhibited high potency whereas clozapine had intermediate potency (Table 2). Two arylpiperidine derivatives, cyproheptadine (a nonselective 5-HT receptor antagonist) and ketanserin (a 5-HT$_2$ receptor antagonist), exhibited weak antagonistic activity (Table 2). Similarly, 1-(1-naphthyl)piperazine was a weak antagonist (Table 2). The indolecarboxylic ester GR 113808 (Fig. 4C) and the azabicycloalkyl benzimidazolone DAU 6285, two selective 5-HT$_4$ receptor antagonists, did not affect the 5-HT-evoked aldosterone secretion.

The effects of three serotonergic antagonists, i.e., mianserin (Fig. 5A–C), methiothepin, and mesulergine, were tested for their ability to inhibit 5-CT-, pergolide-, and 5-HT-induced aldosterone secretion from perifused rat adrenocortical slices. For each antagonist, the pK_B values were not significantly different whatever the agonist used (Table 3).

Pharmacological Characterization of Rat Adrenal 5-HT Receptor. Correlations between the affinities of the drugs for the different serotonergic receptors and their agonistic or antagonistic potencies on aldosterone secretion were examined. No significant correlation ($0.01 < r < 0.52$) was found between the relative affinities of the compounds for the 5-HT$_1$ to 5-ht$_5$ receptors and their effects on aldosterone production (Fig. 6). Similarly, there was no correlation between the affinities of the drugs for the 5-ht$_6$ receptor (Monsma et al., 1993) and their effects on 5-HT-induced aldosterone secretion ($r = 0.49$; $p > .10$) (Fig. 6). In contrast, a significant correlation was observed between the affinities of seven compounds for the 5-HT$_7$ receptor using [^{3}H]LSD as a radioligand (Shen et al., 1993) and their agonistic activity on aldosterone output ($r = 0.82$; $p < .05$) (Fig. 7A). A significant correlation was also found with 10 compounds exhibiting antagonistic activity on 5-HT-induced aldosterone secretion ($r = 0.83$; $p < .01$) (Fig. 7B). In very much the same way, there was a significant correlation between the affinity of the compounds for 5-HT$_7$ receptors labeled with [^{3}H]5-HT (Shen et al., 1993) and their agonistic ($r = 0.79$; $p < .05$) and antagonistic activity ($r = 0.86$; $p < .01$) on aldosterone secretion (data not shown).

Identification of 5-HT$_7$ mRNA in the Rat Adrenal Gland. The presence of 5-HT$_7$ mRNA was investigated by RT-PCR amplification in rat adrenal, brain, and liver. The cDNA of the constitutively expressed housekeeping gene GAPDH was also amplified. A cDNA band of the expected size (963 bp) was readily detected in the reverse transcribed products from the rat adrenal zona glomerulosa and zona fasciculata/reticularis and the rat brain samples (Fig. 8). In the liver, a tissue that does not express the 5-HT$_7$ receptor gene (Lovenberg et al., 1993; Shen et al., 1993), no cDNA band was detected (Fig. 8).

Individual bands obtained from rat adrenal and rat brain reverse transcribed RNA were excised, ligated into pGEM-T, and sequenced. Both sequences corresponded to the published sequences for the 5-HT$_7$ receptors (nt 404-1367) (Lovenberg et al., 1993; Heidmann et al., 1997) (data not shown).

Western Blot Analysis. Western blot analysis was performed to investigate the occurrence of the 5-HT$_7$ receptor

TABLE 1

Effects of various serotonergic receptor agonists on aldosterone secretion from perifused rat adrenal cortex

The effect of each compound on aldosterone secretion was determined as described in *Materials and Methods*. pEC$_{50}$ values and efficacy (E$_{max}$) of the agonists were determined from semilogarithmic concentration-response curves. Hill coefficient (n_H) was calculated for each concentration-response curve. E$_{max}$ is expressed as the net increase in aldosterone secretion (Δpg/min/adrenal). Values are the mean ± S.E. *n* indicates the number of independent experiments.

Compound	pEC$_{50}$	n_H	E$_{max}$	*n*
1. 5-CT	8.23 ± 0.15	0.89 ± 0.11	605.4 ± 37.6	7
2. Pergolide	7.44 ± 0.18	1.07 ± 0.14	679.4 ± 70.6	3
3. 5-HT	7.20 ± 0.13	0.96 ± 0.07	707.6 ± 38.9	17
4. 5-MeOT	5.77 ± 0.21	1.12 ± 0.12	533.2 ± 14.7	8
5. 5-MeODMT	5.69 ± 0.22	0.68 ± 0.05	476.2 ± 66.9	3
6. 8-OH-DPAT	5.47 ± 0.01	1.29 ± 0.27	442.2 ± 65.1	5
7. Tryptamine	<5	0.97 ± 0.36	506.7 ± 33.9	6
(±)-DOI	inactive			3
(*R*,*S*)-zacopride	inactive			3
BIMU 8	inactive			3

protein in the rat adrenal gland. The antiserum revealed the presence of a band with an apparent molecular mass of 66 kDa in both the zona glomerulosa and the zona fasciculata/reticularis samples (Fig. 9). In contrast, no staining was observed in the adrenal medulla and in the liver.

Discussion

The present study has demonstrated that in the rat adrenal gland the stimulatory effect of 5-HT on aldosterone secretion can be accounted for by activation of 5-HT_7 receptors. By using a series of 22 agonists and antagonists, the pharmacological profile of the adrenal serotonergic receptor has been compared with that of the 5-HT_7 receptor transfected in tumor cell lines. The expression of 5-HT_7 receptor mRNA in the rat adrenal cortex has been demonstrated by RT-PCR analysis and the occurrence of the 5-HT_7 receptor protein has been confirmed by Western blotting. The presence of a single receptor population mediating the effect of 5-HT on aldosterone secretion in rat is suggested by the observation that none of the agonists tested produced biphasic concentration-response curves. In addition, the Hill coefficients were close to unity (see Table 1).

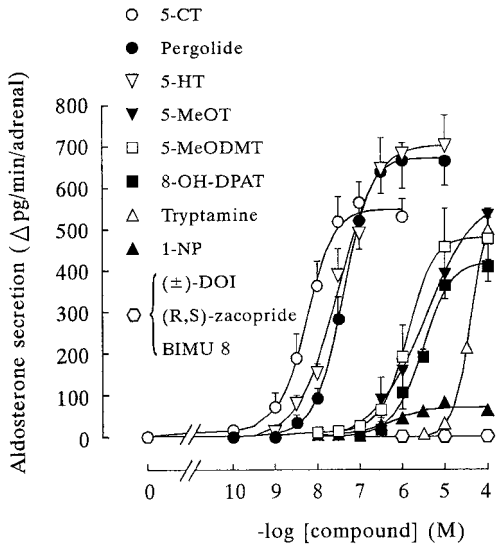

Fig. 2. Concentration-response curves comparing the effects of various agonists on aldosterone secretion by perifused rat adrenocortical slices. Experimental values were calculated from data similar to those presented in Fig. 1A. Each curve represents the mean of at least three independent experiments. See legend to Fig. 1 for other designations. 1-NP, 1-(1-naphthyl)piperazine.

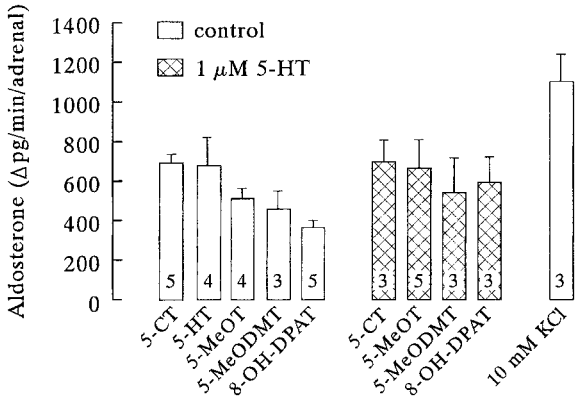

Fig. 3. Effects of serotonergic agonists and 5-HT on aldosterone secretion by perifused rat adrenocortical slices. The effects of 1 μM 5-CT, 10 μM 5-MeOT, 10 μM 5-MeODMT, or 10 μM 8-OH-DPAT on aldosterone secretion were determined independently and in the presence of 1 μM 5-HT. The number of independent experiments is indicated in each column. Experimental values were calculated from data similar to those presented in Fig. 1A.

The adrenal serotonergic receptor exhibited a few pharmacological characteristics in common with the 5-HT_1 receptors. In particular, the 5-HT_1 receptor agonist 5-CT was the most potent stimulator of aldosterone secretion, and the selective 5-HT_{1A} agonist 8-OH-DPAT had a moderate potency in our model. However, the pharmacological profile of the adrenal receptor showed a number of discrepancies with that of the 5-HT_1 receptors. For example, metergoline, an agonist of the 5-HT_{1A}, 5-HT_{1B}, and 5-HT_{1D} receptors (Hoyer et al., 1994) behaved as an antagonist of 5-HT-induced aldosterone secretion. Accordingly, no correlation was found between the affinities of the various ligands at 5-HT_1 receptors with their potencies at the adrenal 5-HT receptor. Similarly, no significant correlation was observed between the pharmacological profile of the adrenal receptor and that of the recently characterized 5-HT_{1G}/5-HT_8 receptor (Castro et al., 1997).

Based on the observation that ketanserin inhibits the stimulatory effect of 5-HT on rat glomerulosa cells, it has been previously purported that the action of 5-HT is mediated by a 5-HT_2 receptor (Williams et al., 1984; Matsuoka et al., 1985). Although we could confirm that ketanserin antagonizes 5-HT-evoked aldosterone secretion, the moderate potency of the compound (Table 2) was not consistent with its high affinity for the 5-HT_{2A} receptor (Leysen et al., 1982). In fact, no correlation was observed ($r = 0.38$) between the binding affinities of a series of ligands for the 5-HT_2 receptors (Hoyer et al., 1994) and their agonistic or antagonistic potencies in our model. Notably, the potent 5-HT_{2A} and 5-HT_{2C} receptor agonist DOI (Nichols et al., 1994) was totally devoid of effect on aldosterone secretion. The fact that the stimulatory effect of 5-HT on aldosterone secretion is mediated through the adenylyl cyclase pathway (Fujita et al., 1979) whereas 5-HT_2 receptors are coupled to phospholipase C (Conn et al., 1986) provides additional evidence that the action of 5-HT on rat glomerulosa cells does not involve 5-HT_2 receptors.

TABLE 2

Effect of various serotonergic receptor antagonists on 5-HT-induced aldosterone secretion from perifused rat adrenal cortex

The effect of each compound was determined as described in *Materials and Methods*. pK_B values of the antagonists were calculated according to Furchgott (1972). Values are the mean ± S.E. *n* indicates the number of independent experiments.

Compound	[B]	pK_B	*n*
8. Methiothepin	0.1 nM	10.13 ± 0.26	8
9. *R*(+)Lisuride	0.1 nM	9.83 ± 0.42	3
R(+)Lisuride	10 nM	10.16 ± 0.14	3
10. Pimozide	0.1 μM	8.75 ± 0.14	6
11. Metergoline	0.1 μM	8.57 ± 0.11	5
12. Clozapine	0.1 μM	8.33 ± 0.01	4
13. Mesulergine	0.1 μM	8.18 ± 0.05	3
14. Mianserin	0.1 μM	7.93 ± 0.11	6
Mianserin	1 μM	7.27 ± 0.25	3
15. Ketanserin	0.1 μM	7.37 ± 0.11	5
16. Cyproheptadine	0.1 μM	7.18 ± 0.13	3
17. 1-(1-Naphthyl)piperazine	0.1 μM	6.92 ± 0.10	3
GR 113808	0.1 μM	<5	3
GR 113808	10 μM	<5	2
DAU 6285	10 μM	<5	2

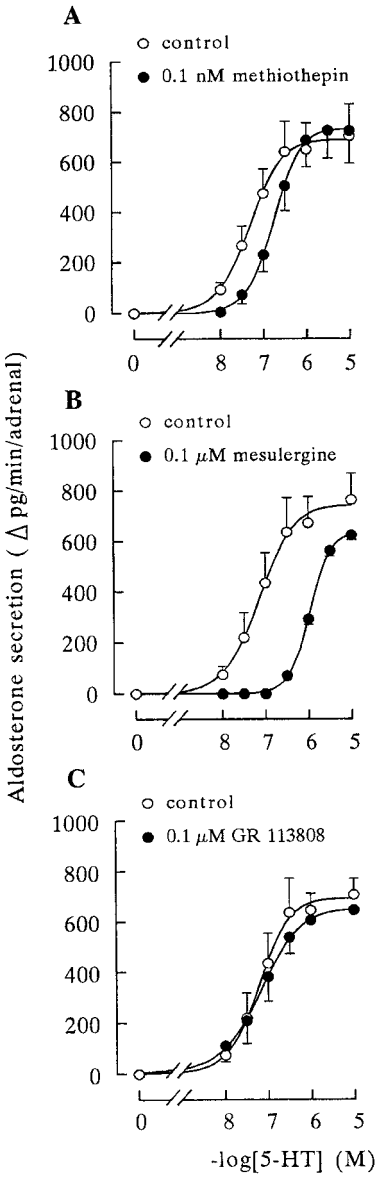

Fig. 4. Effects of three 5-HT receptor antagonists on 5-HT-induced stimulation of aldosterone secretion by perifused rat adrenocortical slices. Concentration response curves of 5-HT on aldosterone secretion in the absence (○) or presence (●) of 0.1 nM methiothepin (A), 0.1 μM mesulergine (B), and 0.1 μM GR113808 (C). Experimental values were calculated from data similar to those presented in Fig. 1A. Each curve represents the mean of at least three independent experiments. See legend to Fig. 1 for other designations.

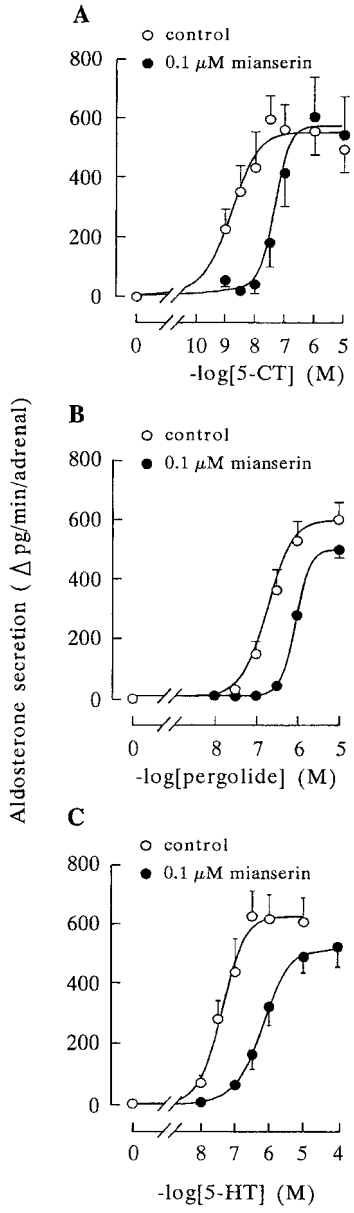

Fig. 5. Effects of mianserin on serotonergic agonist-induced stimulation of aldosterone secretion by perifused rat adrenocortical slices. Concentration response curves of 5-CT (A), pergolide (B), or 5-HT (C) on aldosterone secretion in the absence (○) or presence (●) of 0.1 μM mianserin. Experimental values were calculated from data similar to those presented in Fig. 1A. Each curve represents the mean of at least three independent experiments. See legend to Fig. 1 for other designations.

TABLE 3

Effect of various serotonergic receptor antagonists on agonist-induced aldosterone secretion from perifused rat adrenal cortex

The effect of each compound was determined against the different agonists as described in *Materials and Methods*. pK_B values of the antagonists were calculated according to Furchgott (1972). Values are the mean ± S.E. The number of independent experiments are indicated between parentheses.

Compound	[B]	pK_B Values Obtained Against		
		5-CT	Pergolide	5-HT
Methiothepin	0.1 nM	10.01 ± 0.10 (3)	9.68 ± 0.18 (3)	10.13 ± 0.26 (8)
Mesulergine	0.1 μM			8.18 ± 0.05 (3)
Mesulergine	1 μM	8.04 ± 0.19 (3)		
Mianserin	0.1 μM	7.92 ± 0.17 (3)	7.36 ± 0.22 (4)	7.93 ± 0.11 (6)
Mianserin	1 μM	7.40 ± 0.22 (3)		7.27 ± 0.25 (3)

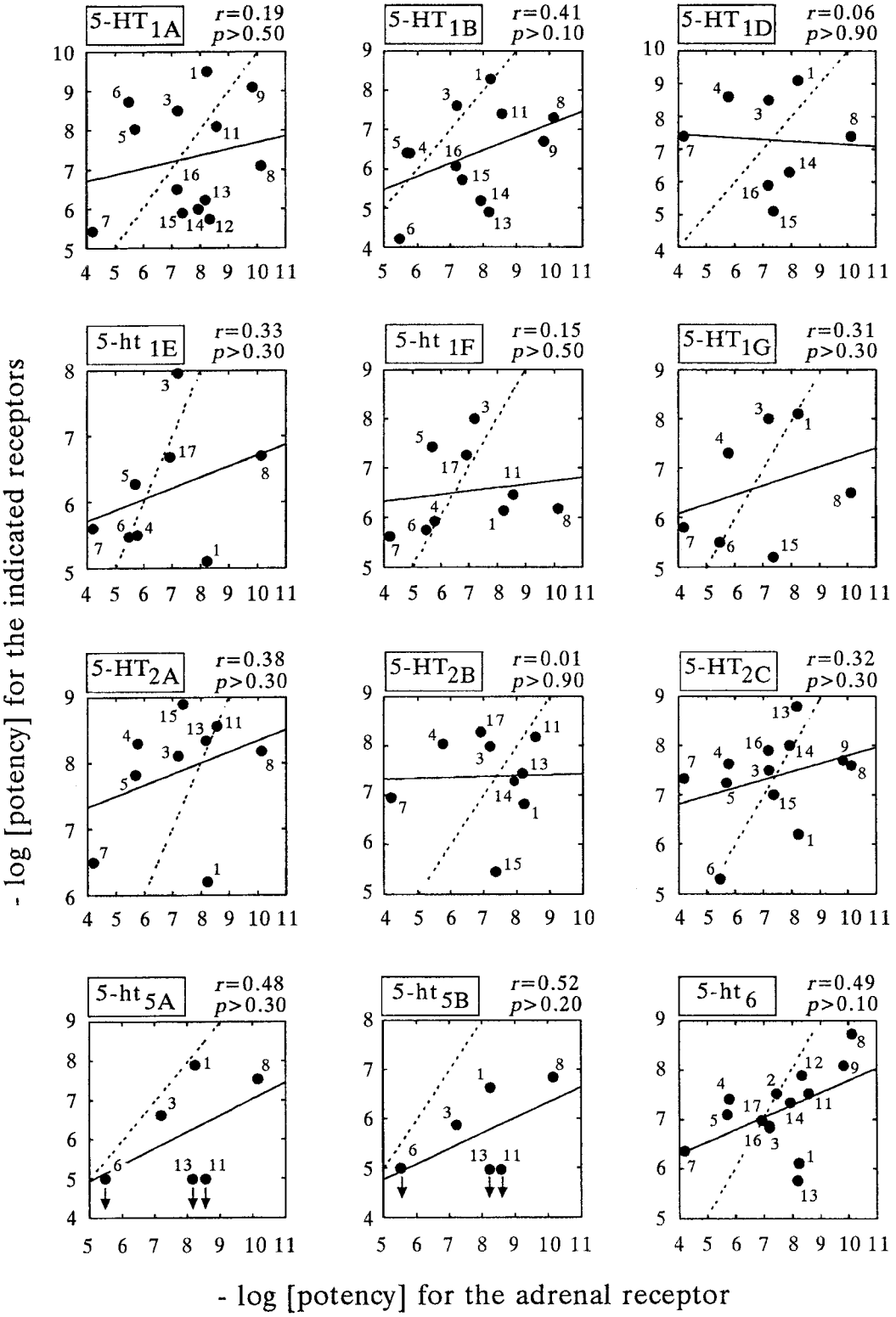

Fig. 6. Correlations between the affinities of various serotonergic ligands for 5-HT receptors and their potencies on aldosterone secretion by the rat adrenal gland. Affinities (pK_i) for 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A}, and 5-HT_{2C} receptors were obtained from Zifa and Fillion (1992). Affinities for 5-HT_{1D}, 5-ht_{1E}, 5-ht_{1F}, and 5-HT_{1G} receptors were obtained from Heuring and Peroutka (1987), Zgombick et al. (1992), Adham et al. (1993), and Castro et al. (1997), respectively. Affinities for 5-HT_{2B} receptors were obtained from Wainscott et al. (1993). Affinities for 5-ht_{5A} and 5-ht_{5B} receptors were obtained from Erlander et al. (1993) and for 5-ht_6 receptors from Monsma et al. (1993). The number beside each point refers to the identification number of the compounds listed in Table 1 and Table 2. Correlation curves were determined by nonweighted linear regression. The arrows indicate that the pK_i was lower than 5. The dotted line represents the line of identity.

The pharmacological profile of the rat adrenal serotonergic receptor is clearly different from that of the 5-HT$_3$ receptor. For instance, the tryptamine derivatives 5-CT and 5-MeOT, which were among the most potent 5-HT agonists on glomerulosa cells are devoid of activity on 5-HT$_3$ receptors (Hoyer et al., 1994). Similarly, the ergolines metergoline and mesulergine, which were found to inhibit 5-HT-induced aldosterone secretion, do not act as 5-HT$_3$ receptor antagonists (Hoyer et al., 1994).

In human and frog, the corticotropic action of 5-HT is mediated through a 5-HT$_4$ receptor (Idres et al., 1991; Lefebvre et al., 1992; Contesse et al., 1996). In these species, the stimulatory effect of 5-HT on aldosterone secretion is mimicked by the 5-HT$_4$ receptor agonists zacopride, cisapride, and BIMU 8 and is abrogated by the 5-HT$_4$ receptor antagonists GR 113808 and DAU 6285 (Lefebvre et al., 1993; Contesse et al., 1994). By contrast, the present study demonstrates that, in rat, 5-HT$_4$ receptors are not involved in the effect of 5-HT on glomerulosa cells: zacopride and BIMU 8 had no effect on aldosterone output whereas GR 113808 and DAU 6285 did not inhibit 5-HT-induced aldosterone secretion. It thus appears that, although 5-HT is a potent stimulator of aldosterone secretion in various vertebrate species, the receptors mediating the effect of 5-HT in rat and human are clearly different.

No significant correlation was observed between the affinities of various serotonergic drugs for the 5-ht$_5$ receptors (Erlander et al., 1993) and their potencies on aldosterone secretion by the rat adrenal gland. In particular, 8-OH-DPAT, which is unable to displace ^{125}I-LSD from recombinant 5-ht$_{5A}$ or 5-ht$_{5B}$ receptors (Erlander et al., 1993), produced a concentration-dependent stimulation of aldosterone secretion. Similarly, metergoline and mesulergine, which are devoid of affinity for 5-ht$_5$ receptors, inhibited 5-HT-induced aldosterone production from the rat adrenal cortex. The fact that 5-ht$_5$ receptors are negatively coupled to adenylyl cyclase (Carson et al., 1996) whereas 5-HT is known to stimulate cAMP formation in the rat adrenal gland (Fujita et al., 1979) provides additional evidence that the effect of 5-HT on aldosterone secretion cannot be ascribed to activation of 5-ht$_5$ receptors.

Among the various serotonergic receptors characterized to date, only the 5-HT$_4$, 5-ht$_6$, and 5-HT$_7$ receptors appear to be positively coupled to the adenylyl cyclase transduction pathway (Dumuis et al., 1989; Monsma et al., 1993; Lovenberg et al., 1993). Because the action of 5-HT on the rat adrenal gland is clearly not mediated through the 5-HT$_4$ receptor (see above), our investigations were focused on the possible involvement of the 5-ht$_6$ and 5-HT$_7$ receptors.

The pharmacological profile of rat adrenal serotonergic receptors is not compatible with that of 5-ht$_6$ receptors transfected in COS-7 cells (Monsma et al., 1993). For example, 5-MeOT, 5-MeODMT, and tryptamine, which exhibit high affinity for recombinant 5-ht$_6$ receptors, were very weak agonists on the rat adrenal receptor. Reciprocally, mesulergine has low affinity for the recombinant 5-ht$_6$ receptor but was relatively potent in antagonizing 5-HT-induced stimulation of aldosterone secretion. As a result, no significant correlation was observed between the potencies of the various serotonergic compounds on rat glomerulosa cells and their K_i values in COS-7 cells transfected with the 5-ht$_6$ receptor (r = 0.49 with ^{125}I-LSD competition assays and r = 0.51 with [^{3}H]5-HT competition assays) (Monsma et al., 1993).

Conversely, a significant correlation was observed between the agonistic or antagonistic activity of the various serotonergic compounds on aldosterone secretion from the rat adrenal cortex (this study) and their binding affinity for 5-HT$_7$ receptors transfected in COS-7, using either [^{3}H]5-HT (r = 0.79, $p < .05$ for agonists; r = 0.86, $p < .01$ for antagonists) or [^{3}H]LSD as radioligands (r = 0.82, $p < .05$ for agonists; r = 0.83, $p < .01$ for antagonists) (Shen et al., 1993). These data strongly suggested that, in rat, the stimulatory effect of 5-HT on aldosterone secretion could be accounted for by activation of 5-HT$_7$ receptors. To test this hypothesis, RT-PCR amplification was conducted using specific oligonucleotide primers for the rat 5-HT$_7$ receptor, and a cDNA fragment of the expected size (963 bp) was generated. Molecular cloning of the PCR product demonstrated that the amplified fragment actually corresponded to the 5-HT$_7$ receptor cDNA sequence. The occurrence of the 5-HT$_7$ protein in the rat adrenal gland was investigated by Western blotting using specific antibodies raised against a synthetic peptide whose sequence corresponds to amino acids 8 to 23 of the rat 5-HT$_7$ receptor. A single band with an apparent molecular mass of 66 kDa was detected in the zona glomerulosa, as well as in the zona fasciculata/reticularis, but not in the adrenal medulla. Although the actual molecular mass of the receptor polypeptide is 45 kDa (Lovenberg et al., 1993; Shen et al., 1993), the 5-HT$_7$ receptor possesses two sites for N-linked glycosylation, suggesting that the adrenal receptor is indeed glycosylated on its N-terminal extracellular segment.

In their pioneer study, Haning et al. (1970) have reported

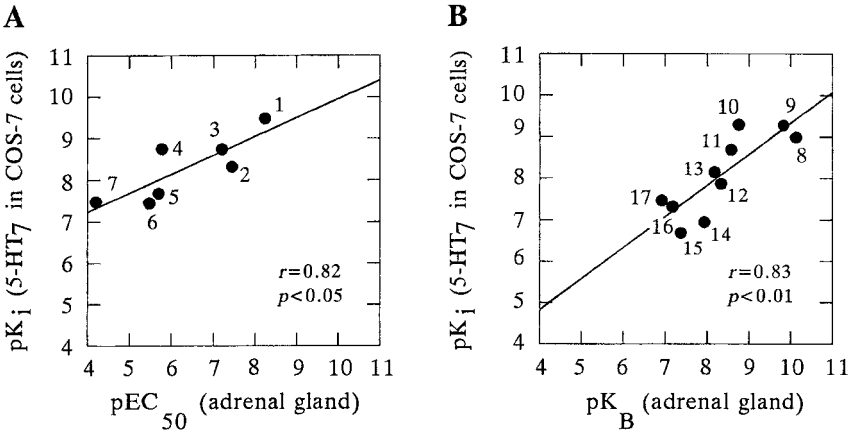

Fig. 7. Correlations between the affinities of various serotonergic agonists (A) and antagonists (B) for the recombinant rat 5-HT$_7$ receptor with the pEC$_{50}$ and pK_B values on aldosterone secretion by the rat adrenal gland. Affinities (pK_i) for the 5-HT$_7$ receptor were obtained from Shen et al. (1993), except for clozapine (no. 12), which was obtained from Roth et al. (1994). See legend to Fig. 6 for other designations.

that 5-HT does not affect corticosterone secretion by the decapsulated portion of the rat adrenal gland (i.e., the adrenal cortex devoid of glomerulosa cells). In contrast, 5-HT causes a weak stimulation of corticosterone secretion (10 times less than aldosterone secretion) from the capsular gland, indicating that 5-HT can only stimulate the production of corticosterone from glomerulosa cells (Haning et al., 1970). We have confirmed this early study (data not shown), which suggests that the 5-HT_7 receptor protein detected by Western blotting in the inner zones of the adrenal cortex is selectively expressed by cells of the zona reticularis. To date, the possible role of 5-HT_7 receptors in the zona reticularis remains totally unknown.

The 5-HT_7 receptor has been initially cloned from the central nervous system (Lovenberg et al., 1993) and has been subsequently localized in several peripheral organs including the gastrointestinal tract and coronary artery (Bard et al., 1993). Pharmacological studies have shown that 5-HT_7 receptors are involved in the action of 5-HT on human uterine artery smooth muscle cells (Schoeffter et al., 1996), *Cynomolgus* monkey jugular vein (Leung et al., 1996), and canine coronary artery (Terrón, 1996). In contrast, to our knowledge, the occurrence of 5-HT_7 receptors has never been described in endocrine glands (Eglen et al., 1997) and this is the first report demonstrating the involvement of 5-HT_7 receptors in the control of hormonal secretions. The presence of 5-HT has been detected by immunohistochemistry in secretory vesicles of rat adrenal chromaffin cells (Lefebvre et al., 1998, for review). Taken together, these data indicate that 5-HT, produced within the adrenal gland, may act as a paracrine factor to regulate locally, through activation of 5-HT_7 receptors, the production of aldosterone.

In summary, the present study indicates that the effect of 5-HT on aldosterone secretion in the rat adrenal gland is mediated through 5-HT_7 receptors. The rat zona glomerulosa is the first endocrine tissue described so far in which a physiological response induced by activation of 5-HT_7 receptors has been investigated. Because the secretion of aldosterone can be readily monitored in vitro, rat glomerulosa cells may prove to be a very suitable model in which to determine whether 5-HT_7 receptor ligands act as agonists or antagonists. This model should be also appropriate to study the regulation of the expression of 5-HT_7 receptors and to investigate the transduction mechanisms associated with activation of native 5-HT_7 receptors.

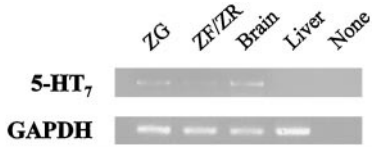

Fig. 8. RT-PCR analysis of 5-HT_7 receptor mRNA in rat tissues. RT-PCR amplification of 5-HT_7 receptor and GAPDH mRNA was performed as described in *Materials and Methods*. Primers specific for 5-HT_7 receptor and GAPDH were used to amplify DNA fragments of 963 and 191 bps, respectively, from zona glomerulosa (ZG), zona fasciculata/reticularis (ZF/ZR) of the rat adrenal gland, brain, and liver. none, no DNA was added to the amplification mixture.

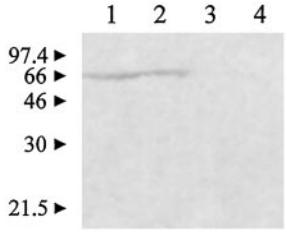

Fig. 9. Western blot analysis of zona glomerulosa (1), zona fasciculata/reticularis (2), medulla (3) from the rat adrenal gland, and liver (4). Tissue samples were homogenized, and proteins (200–300 μg) from 12,000*g* pellets were analyzed by SDS-PAGE followed by immunoblotting with the 5-HT_7 receptor antibodies at a 1:500 dilution. Molecular mass markers (in kDa) are indicated on the left.

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