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Expression and Pharmacological Characterization of a Canine 5-Hydroxytryptamine$_{1D}$ Receptor Subtype

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SUMMARY

RDC4 is a guanine nucleotide-binding protein-coupled receptor clone originally isolated from a canine thyroid cDNA library by Libert and colleagues [*Science (Washington D. C.)* **244**:569–572 (1989)]. We have isolated the corresponding genomic clone for RDC4, have expressed this clone in murine LM (*tk*$^-$) fibroblasts, and have determined that it encodes a serotonin 5-hydroxytryptamine$_{1D}$ (5-HT$_{1D}$) receptor. RDC4 is an intronless gene encoding a protein of 377 amino acids, which exhibits greatest sequence identity (43%) to the 5-HT$_{1A}$ receptor and lower overall homology to other serotonergic and catecholaminergic receptors. Membranes prepared from murine LM (*tk*$^-$) fibroblasts stably transfected with this clone were shown to bind [^{3}H]5-HT in a saturable manner and displayed an apparently homogeneous population of high affinity (K_d = 3.6 nM, B_{max} = 275 fmol/mg of protein) [^{3}H]5-HT binding sites. High affinity [^{3}H]5-HT binding was unchanged using assay conditions [1 μM (±)-pindolol and 1 μM (*R*)-(±)-SCH 23390) to pharmacologically mask 5-HT$_{1A}$, 5-HT$_{1B}$, and 5-HT$_{1C}$ receptors. Serotonergic ligands displaced specific [^{3}H]5-HT binding with a rank order of potency expected of a 5-HT$_{1D}$ receptor subtype, 5-carboxyamidotryptamine > 5-HT > yohimbine > 8-hydroxy-2-(di-*n*-propylamino)tetralin > ketanserin = spiperone > zacopride. Further, transfected cells responded to addition of 5-HT by decreasing the level of forskolin-stimulated cAMP accumulation. These data indicate that the gene RDC4 encodes a functional 5-HT$_{1D}$ receptor.

Serotonin receptors have been classified into four distinct families, designated 5-HT$_1$, 5-HT$_2$, 5-HT$_3$, and 5-HT$_4$, based upon radioligand binding properties, signal transduction mechanisms, and, more recently, deduced amino acid sequences (1, 2). Using these criteria, the 5-HT$_1$ receptor family was further subclassified into four subtypes, termed 5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{1C}$, and 5-HT$_{1D}$, although the 5-HT$_{1C}$ receptor is now considered a member of the 5-HT$_2$ receptor family (2, 3). One recently identified member of this family, the 5-HT$_{1D}$ receptor, was first identified and characterized in bovine caudate homogenates by radioligand binding techniques (4). This receptor subtype was initially defined as a specific [^{3}H]5-HT binding site observed in the presence of 1 μM pindolol and 100 nM mesulergine, to pharmacologically mask 5-HT$_{1A}$, 5-HT$_{1B}$, and 5-HT$_{1C}$ receptors. Compounds exhibiting high affinity for the 5-HT$_{1D}$ receptor subtype include 5-substituted tryptamine derivatives (e.g., 5-CT), ergot alkaloids (e.g., ergotamine), and the α_2-adrenergic antagonists rauwolscine and yohimbine (4–7). With the possible exception of sumatriptan, no selective 5-HT$_{1D}$ ligand has been identified (8). Pharmacological studies have shown the 5-HT$_{1D}$ receptor to be a G protein-coupled receptor, based upon sensitivity of high affinity [^{3}H]5-HT binding to guanine nucleotides (6) and *N*-ethylmaleimide (9) and the requirement for guanine nucleotides for receptor coupling to adenylate cyclase inhibition (10). Interest in the 5-HT$_{1D}$ receptor has intensified because sumatriptan, a selective 5-HT$_{1D}$ agonist (8, 11), has been reported to be effective in the treatment of acute migraine (12).

To date, the genes encoding three serotonin receptor subtypes have been cloned, 5-HT$_{1A}$ (13, 14), 5-HT$_{1C}$ (15, 16), and 5-HT$_2$ (17–19). These receptors belong to a large superfamily of G protein-coupled receptors, which are distinguished by seven TM-spanning domains, an extracellular amino terminus, an intracellular carboxyl terminus, and a third intracellular loop that functionally couples these receptors to signal transduction pathways via G proteins (2, 20). Libert *et al.* (21) have identified a new G protein-coupled receptor (RDC4) that exhibits significant sequence homology to the serotonin 5-HT$_{1A}$ receptor. This clone, along with three others, was isolated by

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; 2-Me-5-HT, 2-methyl-5-hydroxytryptamine; 5-CT, 5-carboxyamidotryptamine; 5-MeOT, 5-methoxytryptamine; 5-MeO-DMT, 5-methoxy-*N*,*N*-dimethyltryptamine; DOI, 1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane; DPAT, 8-hydroxy-2-(di-*n*-propylamino)tetralin; G protein, guanine nucleotide-binding protein; Gpp(NH)p, 5′-guanylylimidodiphosphate; mCPP, 1-(*m*-chlorophenyl)piperazine; PAPP, 1-[2-(4-aminophenyl)ethyl]-4-(3-trifluoromethylphenyl)piperazine; TFMPP, *N*-(*m*-trifluoromethylphenyl)piperazine; TM, transmembrane; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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designing degenerate oligonucleotide primers targeted for the third and sixth TM regions of G protein-coupled receptors, to selectively amplify and clone new members of this superfamily using the polymerase chain reaction. Two of these clones have been functionally expressed and have been reported to encode adenosine A_1 (22, 23) and A_2 receptor subtypes (24). RDC4 has not been fully characterized but is known to exhibit a moderate degree of TM homology (56%) to the human 5-HT$_{1A}$ receptor, suggesting that RDC4 may encode a novel serotonergic receptor. In addition, analysis of critical amino acids that define serotonin receptor subtypes led us to hypothesize that RDC4 encodes a serotonin receptor subtype.[1] In order to determine its identity, we expressed a genomic clone encoding the RDC4 receptor in murine fibroblasts for subsequent pharmacological characterization. The binding properties we observed are consistent with the assignment of this clone as a 5-HT$_{1D}$ receptor subtype.

Materials and Methods

Isolation of RDC4. The RDC4 gene was obtained by isolating clones from a canine genomic library (Stratagene) with an oligonucleotide probe complementary to intracellular loop 3 of the RDC4 gene. Overlapping oligomers complementary to the dog RDC4 sequence (25) were labeled with [^{32}P]dATP and [^{32}P]dCTP by synthesis with the large fragment of DNA polymerase (26). Clones showing positive hybridization to the probe were picked, and inserts were subcloned into the plasmid pUC-18 (Pharmacia, Piscataway, NJ). Sequence analysis confirmed the isolation of a genomic clone containing the entire coding region of the gene, which was identical to the dog cDNA clone (25). The coding region of this gene is not interrupted by introns; however, further investigation of the genomic structure will be needed to determine whether there are introns outside of the coding region.

Expression. A 1.5-kilobase *Hind*III-*Pvu*II genomic DNA fragment containing the entire coding region of RDC4, including 364 base pairs of 5′ untranslated DNA and 50 base pairs of 3′ untranslated DNA, was subcloned into the expression vector pSVL (Pharmacia). Stable cell lines were generated by co-transfection with plasmids pSVL (containing the RDC4 gene) and pGCcos3neo, using the calcium phosphate method. Cells were grown as monolayers and selected for antibiotic resistance (27). Stable transfectants were screened for specific binding of [^{3}H]5-HT, and one clonal cell line, RDC4-14, was selected for pharmacological characterization. In initial experiments, membranes prepared from RDC4-14 were tested for binding of [^{3}H]5-HT under 5-HT$_{1D}$ assay conditions. Specific [^{3}H]5-HT binding remained the same in the absence and in the presence of masking ligands [1 μM (±)-pindolol and 1 μM (*R*)-(+)-SCH-23390]. Therefore, masking ligands were omitted in all subsequent assays using membranes derived from this clonal cell line. No specific [^{3}H]5-HT binding was detected to membranes from sham-transfected cells. Cells were grown as monolayers to 100% confluency before membranes were harvested for binding assays (27).

Membrane preparation. Membranes for radioligand binding assays were prepared from stably transfected cells, as described previously (28). Freshly prepared membranes were used within 1 hr of preparation for all radioligand binding assays. To best compare the binding properties of RDC4 with the pharmacologically defined 5-HT$_{1D}$ receptor, bovine striatal tissue was used as a model of this receptor subtype. Fresh bovine brains were obtained from a local slaughterhouse. Striata were dissected and membranes for binding assays were prepared by the method of Herrick-Davis and co-workers (5, 6).

Radioligand binding studies. [^{3}H]5-HT binding was performed using 5-HT$_{1D}$ assay conditions, in the absence (clonal cell membranes) and presence (bovine striatal membranes) of masking ligands (5, 6). Radioligand binding studies were conducted in 96-well microtiter plates, in a total volume of 250 μl of buffer (50 mM Tris, 10 mM $MgCl_2$, 0.2 mM EDTA, 10 μM pargyline, 0.1% ascorbate, pH 7.4 at 37°). Occupancy studies were conducted using [^{3}H]5-HT concentrations ranging from 0.25 to 70 nM, and competition studies were performed using 5 nM [^{3}H]5-HT. Unlabeled 5-HT (10 μM) was used to define nonspecific binding. The binding assay was initiated by addition of 50 μl of membrane homogenate (1.5 μg/μl) and was incubated at 37° for 30 min in the dark. The reaction was terminated by rapid filtration through presoaked (0.5% polyethyleneimine) filters, using a Brandel 48R cell harvester (Gaithersburg, MD). Filters were washed for 5 sec with ice-cold buffer (50 mM Tris·HCl, pH 7.4 at 4°). Dried filters were placed in vials containing 2.5 ml of Ready-Safe (Beckman, Fullerton, CA), and radioactivity was measured using a Beckman LS 5000TA liquid scintillation counter. The counting efficiency of [^{3}H]5-HT averaged 50%. Protein concentrations were determined by the method of Bradford (29), using bovine serum albumin as the standard.

Functional studies. Intracellular cAMP levels were measured using the clonally derived cell line described above. Cells were preincubated for 20 min at 37° in 5% CO_2, in Dulbecco's modified Eagle's medium containing 10 mM HEPES, 5 mM theophylline, and 10 μM pargyline. Methiothepin (10 μM final concentration) was also included during this preincubation in antagonist experiments. Cells were incubated with forskolin (10 μM final concentration) and/or 5-HT (1 μM final concentration) for an additional 10 min at 37° in 5% CO_2. The assay was terminated by the removal of medium and the addition of 500 μl of 0.1 M HCl. Cells were processed as previously described,[1] and intracellular cAMP levels were determined by radioimmunoassay (Advanced Magnetics, Cambridge, MA).

Data analysis. Binding data were analyzed by computer-assisted nonlinear regression analysis (ACCUFIT and ACCUCOMP; Lundon Software, Chagrin Falls, OH). IC_{50} values were converted to K_i values using the Cheng-Prusoff equation (30). Linear regression was used to determine correlation between parameters. All experiments were performed a minimum of three times.

Drugs. Drugs were obtained from the following companies: [^{3}H]5-HT (specific activity, 20.4–28.0 Ci/mmol), New England Nuclear (Boston, MA); 5-HT, ergotamine, (±)-pindolol, and Gpp(NH)p, Sigma (St. Louis, MO); 5-CT, 5-MeOT, DPAT, 5-Me-O-DMT, PAPP, TFMPP, mCPP, 2-Me-5-HT, DOI, methysergide, (−)-propranolol, spiperone, yohimbine, and (*R*)-(+)-SCH-23390, Research Biochemical Inc. (Natick, MA); rauwolscine, Accurate Chemicals (Westbury, NY); methiothepin, Biomol Research Laboratories (Plymouth Meeting, PA); and forskolin, Calbiochem (La Jolla, CA). All other chemicals were the highest purity available commercially.

Results

The deduced amino acid sequence of RDC4 shares considerable sequence homology with other biogenic amine receptors. A variety of structural features that are invariant in this family include the aspartic acid residues of TM regions II and III, the DRY sequence at the end of TM region III, and the conserved proline residues of TM regions IV, V, VI, and VII (2, 20). The amino acid sequence of RDC4 displayed greatest homology with the 5-HT$_{1A}$ receptor and lower overall identity to other serotonergic (5-HT$_{1C}$ and 5-HT$_2$) and catecholaminergic receptor sequences (Table 1). This structural information suggested that RDC4 may be a member of the large family of serotonin receptors. We have, therefore, cloned the RDC4 gene from a canine genomic library and expressed it in murine fibroblasts, in order to determine the pharmacological binding properties of this receptor.

An intronless genomic clone designated RDC4 was stably

[1]Weinshank, R. L., Zgombick, J. M., Macchi, M. J., Branchek, T. A., and Hartig, P. R. The human serotonin 1D receptor is encoded by a subfamily of two distinct genes: 5-HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$. *Proc. Natl. Acad. Sci. USA*, in press.

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TABLE 1

Comparison of the deduced amino acid sequence of RDC4 with other cloned biogenic amine receptors

Overall identity indicates identical amino acids between the receptor pair.

Cloned receptor	Overall identity
	%
α_{1A}	32
α_{1B}	36
α_{1C}	37
α_{2A}	39
α_{2B}	34
α_{2C}	37
β_1	36
β_2	36
β_3	32
D_1	38
D_2	36
D_3	38
5-HT_{1A}	43
5-HT_{1C}	32
5-HT_2	30

expressed in murine LM (tk^-) cells, and membranes harvested from these cells were tested for their ability to specifically bind [^{3}H]5-HT. In initial experiments, specific [^{3}H]5-HT binding was evaluated under 5-HT_{1D} assay conditions. In the presence of 1 μM (±)-pindolol to mask 5-HT_{1A} and 5-HT_{1B} sites and 1 μM (*R*)-(+)-SCH-23390 to mask 5-HT_{1C} sites, specific [^{3}H]5-HT binding was unchanged (1446 cpm versus 1487 cpm), suggesting that RDC4 encodes a 5-HT_{1D} receptor. Membranes prepared from cells transfected with the RDC4 gene displayed an apparently homogeneous population of high affinity (K_d = 3.6 nM), saturable (B_{max} = 275 fmol/mg of protein), [^{3}H]5-HT binding sites (Fig. 1). Specific [^{3}H]5-HT binding was >75% of total [^{3}H]5HT binding at the K_d value. No specific [^{3}H]5-HT binding was observed in untransfected or mock-transfected host cells.

The pharmacological identity of RDC4 was determined from analysis of competition of [^{3}H]5-HT binding. Serotonergic agonists and antagonists displaced specific [^{3}H]5-HT binding in a monophasic manner, with Hill coefficients not deviating significantly from unity ($n_H \sim 1$; Table 2), consistent with the labeling of an apparently homogeneous population of noninteracting binding sites. The rank order of potency of these ligands (Table 2) to compete for the [^{3}H]5-HT-labeled binding site is consistent with a 5-HT_{1D} receptor pharmacology, 5-CT > 5-HT > yohimbine > DPAT > ketanserin = spiperone > zacopride. Compounds exhibiting high affinity for RDC4 and the pharmacologically defined 5-HT_{1D} receptor in native brain tissue include tryptamine (5-CT, 5-HT, and 5-MeOT) and ergoline (ergotamine and methysergide) derivatives and α_2-adrenergic antagonists (rauwolscine and yohimbine). Sumatriptan, a reportedly selective 5-HT_{1D} ligand (8, 11), was one of the most potent compounds at this site (K_i = 1.6 nM). Because this affinity value was higher than that previously reported for bovine striatal membranes, we determined the K_i of sumatriptan in our laboratory. We found that sumatriptan showed an affinity constant of 40 ± 3 nM in that tissue preparation. Subtype-selective drugs that displayed low affinity (>80 nM) were DPAT (5-HT_{1A} agonist), spiperone (5-HT_{1A}/5-HT_2 antagonist), pindolol (5-HT_{1A}/5-HT_{1B} antagonist), ketanserin (5-HT_2 antagonist), and zacopride (5-HT_3 antagonist; Table 2). The endogenous catecholamines (dopamine, norepinephrine, and epinephrine) were all inactive.

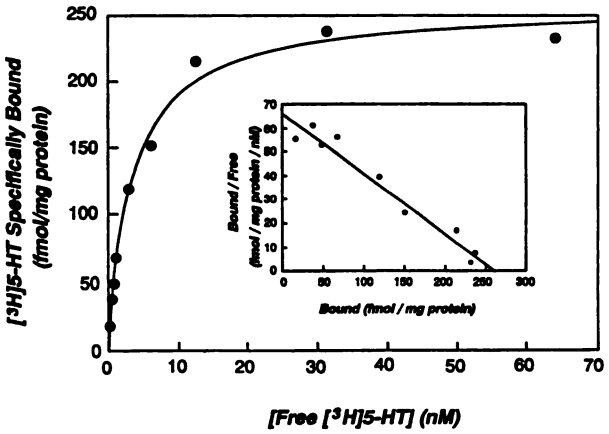

Fig. 1. Occupancy curve for specific [^{3}H]5-HT binding to LM (tk^-) membranes stably expressing RDC4. Membranes prepared from LM (tk^-) cells stably transfected with RDC4 were incubated with increasing concentrations of [^{3}H]5-HT (0.25–70 nM), in the absence and presence of 10 μM unlabeled 5-HT, for 30 min at 37°. Each data point represents the mean of triplicate determinations, and standard deviations averaged <5%. Estimates of K_d and B_{max} were determined by nonlinear regression analysis and were used in the Scatchard plot (*inset*). Estimates for K_d and B_{max} values from this experiment were 3.6 nM and 265 fmol/mg of protein, respectively.

In order to compare the binding properties of RDC4 with those of the pharmacologically defined 5-HT_{1D} receptor, competition studies were performed using bovine striatum as a model tissue for this receptor subtype. In these experiments, 5-HT_{1D} assay conditions were used to mask 5-HT_{1A}, 5-HT_{1B}, and 5-HT_{1C} receptors. The equilibrium dissociation constant of [^{3}H]5-HT (K_d = 5.5 nM) and site density (B_{max} = 300 fmol/mg of protein) are similar to values obtained with RDC4 (Fig. 1) and reported literature values (4–7). Moreover, the rank order of potency of ligands to displace specific [^{3}H]5-HT binding in bovine striatal homogenates was congruent with previous descriptions of the 5-HT_{1D} site (4–7) and matched that of RDC4 (Table 2). Correlations were calculated between the affinity constants (pK_i values) of these serotonergic ligands obtained with cells stably expressing RDC4 and previously published values for 5-HT_1 receptor subtypes in native brain membranes. A high degree of correlation (r = 0.84) was obtained between RDC4 and the 5-HT_{1D} receptor subtype. Other 5-HT receptor subtypes showed significantly lower correlation coefficients (Fig. 2).

The functional coupling of RDC4 to adenylate cyclase inhibition was determined in intact cells stably expressing this gene (Fig. 3). Forskolin (10 μM) produced a 3–5-fold elevation in cAMP levels above basal values. 5-HT (1 μM) inhibited forskolin-stimulated cAMP accumulation by 32 ± 1% in transfected cells. The nonselective antagonist methiothepin (1 μM) completely blocked the response to 5-HT. In the absence of forskolin, 5-HT did not stimulate adenylate cyclase in these transfected cells. In untransfected cells, 5-HT did not stimulate or inhibit cAMP production (data not shown).

Discussion

The deduced amino acid sequence of RDC4 was compared with protein sequences of previously cloned G protein-coupled receptors, to evaluate its relationship to other members of this superfamily. As previously noted (21), RDC4 exhibits greatest overall identity (43%) to the 5-HT_{1A} receptor and lower identity

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TABLE 2

Potency (K_i values) of serotonergic ligands to displace specific [^{3}H]5-HT binding to membranes of LM (tk$^-$) cells stably expressing the cloned canine 5-HT$_{1D}$ receptor (RDC4) and membranes from bovine striatum containing the pharmacologically defined 5-HT$_{1D}$ receptor

Membranes prepared from stable transfectants or bovine striatum were incubated with 5 nM [^{3}H]5-HT, in the absence and presence of 10 concentrations of unlabeled competitor, for 30 min at 37°. Unlabeled 5-HT (10 μM) was used to define nonspecific binding. IC_{50} values were converted to K_i values using the Cheng-Prusoff equation (30). K_i values and Hill coefficients are expressed as mean ± standard error from three to nine determinations. Affinity constants of some compounds at the 5-HT$_{1D}$ receptor in bovine striatal membranes were obtained from literature values (8, 33, 46).

Compound	RDC4		
	K_i	Hill coefficient	Bovine striatum K_i
	nM		nM
5-CT	0.92 ± 0.06	0.83 ± 0.05	4.6 ± 1.4
Sumatriptan	1.62 ± 0.18	0.89 ± 0.04	17[a]
Ergotamine	1.94 ± 0.29	1.22 ± 0.16	14 ± 4.0
5-HT	5.0 ± 1.4	0.83 ± 0.05	6.4 ± 1.4
5-MeO-DMT	5.1 ± 1.7	0.80 ± 0.09	32[b]
Rauwolscine	5.3 ± 2.9	1.12 ± 0.19	41 ± 2
Methysergide	7.7 ± 1.7	0.80 ± 0.01	25 ± 1
5-MeOT	31 ± 11	0.88 ± 0.14	26 ± 8
Yohimbine	32 ± 6	0.87 ± 0.05	32 ± 3
PAPP	35 ± 6	1.14 ± 0.19	7.6[c]
DPAT	86 ± 21	0.89 ± 0.09	628 ± 59
TFMPP	112 ± 14	0.97 ± 0.27	600
mCPP	329 ± 80	0.94 ± 0.07	2,800
Tryptamine	343 ± 15	0.85 ± 0.02	300
2-Me-5-HT	444 ± 30	0.80 ± 0.10	398[b]
DOI	729 ± 102	0.83 ± 0.06	6,116 ± 797
Ketanserin	>1,000	ND[d]	1,000[b]
Spiperone	>1,000	ND	5,010[b]
(−)-Propranolol	>10,000	ND	3,162[b]
(±)-Pindolol	>10,000	ND	6,310[b]
Zacopride	>10,000	ND	ND
Norepinephrine	>10,000	ND	ND
Epinephrine	>10,000	ND	ND
Dopamine	>10,000	ND	ND

[a] From Ref. 8.
[b] From Ref. 33.
[c] From Ref. 46.
[d] ND, not determined.

(<40%) to other serotonergic and catecholaminergic receptors (Table 1). The moderate degree of TM homology between RDC4 and the 5-HT$_{1A}$ receptor initially led Libert *et al.* (21) to speculate that this clone encoded a serotonin receptor. More detailed analysis of amino acids characteristic of serotonin receptor clones provided additional support for this hypothesis.

In order to determine the receptor subtype encoded by RDC4, a genomic RDC4 was stably expressed in murine fibroblasts, for subsequent pharmacological characterization. The binding properties of the receptor encoded by RDC4 best matched the properties of a 5-HT$_{1D}$ receptor subtype. Several lines of evidence support this idea. The high affinity [^{3}H]5-HT binding (K_d = 3.6 nM; Fig. 1) observed in the presence of 1 μM (±)-pindolol (to mask 5-HT$_{1A}$ and 5-HT$_{1B}$ receptor sites) and 1 μM (*R*)-(+)-SCH 23390 (to mask 5-HT$_{1C}$ receptor sites) would exclude the possibility that the radioligand was labeling these pharmacologically defined receptor subtypes. Additionally, the nanomolar affinity of [^{3}H]5-HT for RDC4 (K_d = 3.6 nM; Fig. 1) satisfies a pharmacological criterion for classification as a member of the 5-HT$_1$ receptor family (3). The rank order of potency of ligands to displace specific [^{3}H]5-HT binding in membranes prepared from cells stably expressing RDC4 (5-CT > 5-HT > yohimbine > DPAT ≫ pindolol = spiperone = ketanserin > zacopride) closely matches the 5-HT$_{1D}$ receptor pharmacology obtained using bovine striatal homogenates (Table 2). Compounds exhibiting high affinity for the receptor encoded by RDC4 and for the 5-HT$_{1D}$ receptor (Table 2) include tryptamine derivatives (5-CT, 5-HT, and 5-MeOT), ergot alkaloids (ergotamine and methysergide), and α_2-adrenergic antagonists (rauwolscine and yohimbine) (4–7). Moreover, sumatriptan, a reported 5-HT$_{1D}$-selective ligand (8), exhibited very high affinity for this site (K_i = 1.6 nM), greater than that of 5-HT itself (Table 2). When measured in our laboratory, sumatriptan displayed a much lower affinity in bovine brain (K_i = 40 nM) than that measured for the cloned receptor. This value was in reasonable agreement with that reported for the bovine caudate (29 nM) (11). These data indicate potentially important species differences in the affinity of this drug.

To further support the hypothesis that RDC4 encodes a 5-HT$_{1D}$ receptor, correlations of ligand affinities (pK_i values) were obtained between binding data derived from RDC4 and from the pharmacologically defined 5-HT$_1$ receptor subtypes (Fig. 2). The highest correlation (r = 0.84) was obtained between RDC4 and the 5-HT$_{1D}$ receptor subtype; much lower correlations ($r \leq 0.45$) were observed between RDC4 and the other 5-HT$_1$ subtypes (Fig. 2). The 5-HT$_{1B}$ subtype can be excluded, because 5-HT$_{1B}$ receptors have not been found in higher mammalian species (31) and the binding properties reported here do not agree with those of a 5-HT$_{1B}$ binding site (32, 33). The poor correlation of ligand affinities between RDC4 and the 5-HT$_{1C}$ receptor (r = 0.16; Fig. 2) would rule out the possibility that RDC4 encodes a 5-HT$_{1C}$ receptor subtype. RDC4 cannot be considered a 5-HT$_{1A}$ receptor subtype, based upon the low affinity of a structurally diverse collection of 5-HT$_{1A}$ ligands, including DPAT, PAPP, spiperone, propranolol, and pindolol, as well as the high affinity of sumatriptan and

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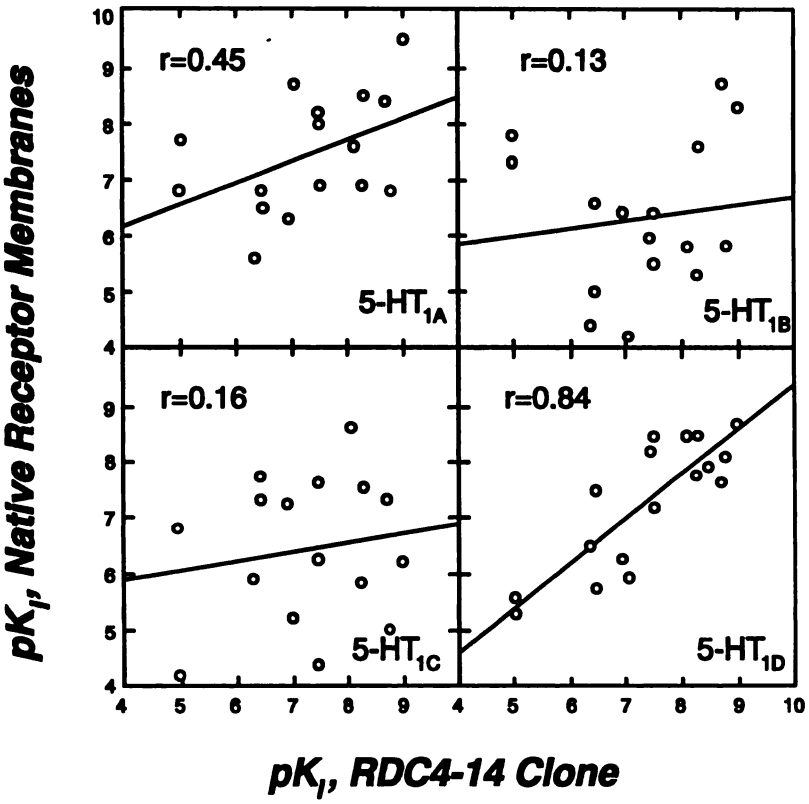

Fig. 2. Correlations between the pK_i values of serotonergic ligands for RDC4 and four pharmacologically defined $5HT_1$ receptor subtypes. The pK_i values of ligands for the $5\text{-}HT_1$ subtypes were taken from literature values (8, 33, 46). The correlation coefficient (*r*) is listed in each panel.

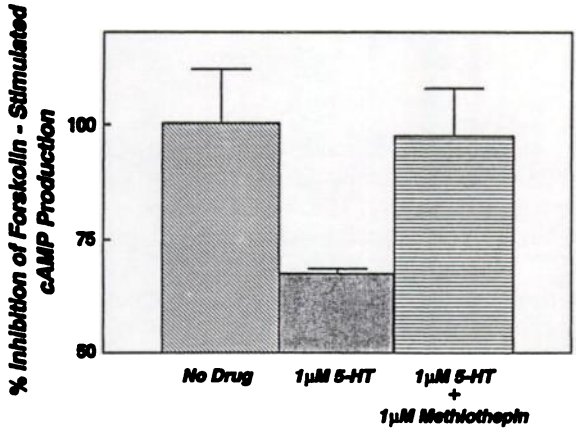

Fig. 3. Inhibition of forskolin-stimulated cAMP production by 5-HT and antagonism by methiothepin in cells stably expressing the RDC4 gene. Forskolin was used at a final concentration of 10 μM. Experimental conditions are listed below each *bar*. Data represent mean values ± standard errors from triplicate determinations from a representative experiment. The experiment was replicated an additional two times, with similar results.

the α_2-adrenergic antagonists (rauwolscine and yohimbine) (Table 2).

Pharmacological studies have shown the $5\text{-}HT_{1D}$ receptor to be a G protein-coupled receptor, based upon sensitivity of high affinity [^{3}H]5-HT binding to guanine nucleotides (6) and *N*-ethylmaleimide (9) and the requirement for guanine nucleotides for receptor coupling to adenylate cyclase inhibition (10). In preliminary studies, Gpp(NH)p reduced specific [^{3}H]5-HT binding by nearly 90%, with an IC_{50} value of 1 μM. These values are similar to those obtained for the $5\text{-}HT_{1D}$ receptor in native brain membranes (6). In addition, second messenger studies were performed using a cell line stably expressing the RDC4 gene (RDC4-14). 5-HT, applied to these intact cells, produced a decrease in cAMP accumulation in response to forskolin (Fig. 3). This response was blocked by methiothepin. These results indicate that RDC4 encodes a functional serotonin receptor.

The $5\text{-}HT_{1D}$ receptor has been found in the brain and peripheral tissues of a variety of mammalian species, including human, where it has been shown to mediate a variety of functional responses (10, 34–38). Comparison of agonist efficacies between these functional preparations reveals differences in the rank order of agonist potencies and apparent receptor heterogeneity. These variations in agonist potency may result from receptor and/or G protein reserve in model tissues (39, 40), species variants of the same receptor subtype imparting different pharmacologies (41), or the existence of multiple $5\text{-}HT_{1D}$ receptor subtypes (42).

Recently, our group has cloned two human $5\text{-}HT_{1D}$ receptor subtypes, designated $5\text{-}HT_{1D\alpha}$ and $5\text{-}HT_{1D\beta}$, which possess strong similarities in protein sequence, pharmacological properties, and second messenger coupling (43).[1] Comparison of the deduced amino acid sequence of RDC4 and one of the human clones ($5\text{-}HT_{1D\alpha}$) reveals overall homology of 87% and TM homology of 92%.[1] This high degree of amino acid conservation, particularly in the TM-spanning domains, is typical of genes that encode species homologs of the same receptor subtype. Examples illustrating the high sequence identity (>90% in the TM regions) between the rat and human versions of other G protein-coupled receptors include the $5\text{-}HT_{1A}$ (13, 44), $5\text{-}HT_2$ (17, 18), and α_{2B}-adrenergic (27, 45) receptor subtypes. Based upon these analyses, we conclude that RDC4 and $5\text{-}HT_{1D\alpha}$ represent species homologs of the same gene, which encode the same $5\text{-}HT_{1D}$ receptor subtype in different species (canine versus human).

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In conclusion, RDC4 was predicted to encode a novel serotonergic receptor, on the basis of the comparison of its deduced amino acid sequence with that of other biogenic amine (G protein-coupled) receptors and analysis of critical amino acids that define serotonin receptor subtypes. We determined that RDC4 encodes a 5-HT$_{1D}$ receptor subtype, based upon its pharmacological binding properties, including its high affinity for [^{3}H]5-HT under 5-HT$_{1D}$ assay conditions, its rank order of ligand potency to compete for this labeled site (Table 2), the high affinity of certain α_2-adrenergic antagonists (rauwolscine and yohimbine) and 5-HT$_{1D}$-selective ligands (e.g., sumatriptan) for this site, and its functional coupling to the inhibition of adenylate cyclase. The relationship of RDC4 to the array of 5-HT$_{1D}$-like receptors characterized pharmacologically is not known at present. Further studies will determine the relationship of this clone to these pharmacological sites and should lead to the cloning of all members of this new serotonin receptor subfamily.

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