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The Cloning and Expression of an OK Cell cDNA Encoding a 5-Hydroxytryptamine$_{1B}$ Receptor

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SUMMARY

Serotonin (5-hydroxytryptamine or 5-HT) is an important biogenic amine that functions as both a neurotransmitter and a hormone in the central nervous system (CNS) and the periphery. We report here the isolation of a cDNA from the OK cell that encodes a serotonin receptor (OKc1). When expressed in cultured cells, it displayed the pharmacological profile and negative coupling with adenylyl cyclase characteristic of a 5-HT$_{1B}$ receptor subtype. Similar to the cloned rodent 5-HT$_{1B}$ receptors, it had high affinity for the β-adrenergic ligand [^{125}I]iodocyanopindolol, because of the presence of an asparagine instead of a threonine residue in the seventh transmembrane region. The ligands used displayed the following rank order of potencies: cyanopindolol > RU24969 > methiothepin > serotonin > sumatriptan > methysergide > 8-OH-DPAT > isoproterenol. This profile correlates well (r = 0.97) with the native OK cell 5-HT$_{1B}$ receptor. When OKc1 is compared to the rat, mouse, and human 5-HT$_{1B}$ receptors, it has an amino acid sequence identity of 82%, but it is only 54% identical to the human 5-HT$_{1D}$ receptor.

Serotonin is an important biogenic amine that functions as both a neurotransmitter and a hormone in the mammalian CNS and in the periphery. Many serotonergic drugs are psychoactive, and serotonin has been implicated in a variety of mental disorders, including depression and panic disorder, as well as being involved in anxiety, appetite regulation, and sleep. In the periphery, 5-HT plays a major role as an effector in systems as diverse as the cardiovasculature and the gastrointestinal system.

Previous classification schemes based originally on pharmacological characteristics and effector coupling mechanisms recognized three major types of 5-HT receptors: 5-HT$_1$, 5-HT$_2$, and 5-HT$_3$ (1, 2). Molecular biological data have now both confirmed and expanded the number of 5-HT receptors known. 5-HT$_1$ and 5-HT$_2$ receptors belong to the G protein receptor superfamily and mediate most, if not all, their effects by coupling to guanine nucleotide regulatory binding proteins (G proteins). Further heterogeneity exists within the 5-HT$_1$ receptor as well as several 5-HT$_2$ receptors. One of the 5-HT$_1$ receptors, 5-HT$_{1C}$, now is considered by most investigators to be more properly classified with the 5-HT$_2$ receptors (3, 4). The 5-HT$_2$ receptor family contains the 5-HT$_2$ receptors (rat, mouse, hamster, and human) (5–8), the 5-HT$_{1C}$, now designated the 5-HT$_{2C}$ receptor (4, 8), and the 5-HT$_{2F}$ receptor (9). In contrast to the 5-HT$_1$ receptors, which are coupled to adenylyl cyclase, the 5-HT$_2$ receptors activate phospholipase C.

The 5-HT$_1$ receptor type currently is subdivided into the 5-HT$_{1A}$, 5-HT$_{1B}$ (5-HT$_{1D\beta}$), 5-HT$_{1D}$ (5-HT$_{1D\alpha}$), 5-HT$_{1E}$, and 5-HT$_{1F}$ subtypes, all of which are coupled to the inhibition of adenylyl cyclase. The 5-HT$_{1A}$ receptor was the first serotonin receptor to be cloned. It was isolated from a human genomic library using a β_2-adrenergic receptor probe and encoded an intronless 421-amino-acid protein with the pharmacological properties of a 5-HT$_{1A}$ receptor (10, 11). The homologous rat receptor has also been cloned and shows 99% sequence similarity to the human receptor in the TM regions (12). The first 5-HT$_{1B}$ receptor to be cloned was from a rat brain cDNA library (13) using primers derived from RDC4 (14), a canine G-linked receptor clone showing high homology to the serotonin receptors and thought at the time to encode a new 5-HT receptor subtype. This was followed by the cloning of the mouse (15) and the human 5-HT$_{1B}$ (or 5-HT$_{1D\beta}$) orthologues (16–20). Overall, the rodent receptors are about 93% homologous to the human receptor, with 96% amino acid identity in the TM regions.

The 5-HT$_{1B}$ receptors have approximately 60% sequence identity to the 5-HT$_{1D}$ receptor subtype. Libert *et al.* (14) cloned

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ABBREVIATIONS: CNS, central nervous system; 5-HT, 5-hydroxytryptamine; RU24969, 5-methoxy-3-(1,2,3,6-tetrahydro-4-pyridinyl)1H-indol; 8-OH-DPAT, 8-hydroxydipropylaminotetralin; G protein, guanine nucleotide regulatory binding protein; TM, transmembrane spanning; SDS, sodium dodecyl sulfate; RACE, rapid amplification of cDNA ends; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TCA, trichloroacetic acid; DMSO, dimethylsulfoxide; DEAE, *O*-(diethylaminoethyl); OKc1, OK cell cDNA clone encoding a 5-HT$_{1B}$ receptor; CHO, Chinese hamster ovary; PCR, polymerase chain reaction; kb, kilobase; bp, base pair; BSA, bovine serum albumin.

the first example of this receptor subtype, RDC4, later confirmed as a 5-HT$_{1D}$ receptor (21, 22). This was followed by the human (23) and rat (24) species orthologues. The human gene, designated 5-HT$_{1D}$ (or 5-HT$_{1D\alpha}$) has 90% sequence identity with the rat gene and 87% identity with the canine gene (92% in the TM regions). The 5-HT$_{1E}$ subtype, referred to as S31 or AC1, is a 365-amino-acid protein with a structure typical of G-linked receptors and has been cloned from the human (25, 26). The last of the cloned 5-HT$_1$ receptors, the 5-HT$_{1F}$ subtype, has been isolated from both the mouse (27, 28) and the human (28, 29). These receptors display the characteristic seven TM regions of the other cloned mammalian serotonin receptors. The mouse receptor exhibits 61% identity to the 5-HT$_{1E}$ receptor. The human 5-HT$_{1F}$ gene shares 94% amino acid identity with its mouse homologue. Additionally, several *Drosophila* serotonin receptors have been cloned, including 5-HT-dro-1 (30), which activates adenylate cyclase, and 5-HT-dro-2A and 5-HT-dro-2B, which are coupled negatively to adenylyl cyclase (31).

The 5-HT$_3$ receptors are members of the ligand-gated ion channel superfamily of receptors. The recent pharmacologically characterized 5-HT$_4$ or 5-HT$_4$-like receptors (32, 33) that mediate vascular relaxation appear to be G protein-coupled receptors associated with stimulation of adenylyl cyclase. The 5-HT$_4$ receptor has not been cloned yet.

The 5-HT$_{5A}$ and 5-HT$_{5B}$ receptors recently have been added to the serotonin receptor family (34–36). The pharmacological profiles of the mouse 5-HT$_{5A}$ and 5-HT$_{5B}$ receptors are similar, with low affinity for 5-HT, but high affinity for 5-CT and a low affinity for sumatriptan. The 5-HT$_{5A}$ and 5-HT$_{5B}$ receptors share 77% sequence similarity, and both genes contain introns. Coupling of 5-HT$_{5A}$ to either adenylyl cyclase or phospholipase C has not been detected, and it is possible that both receptors couple to ion channels.

Another recent addition to the serotonin family is a novel serotonin receptor with high affinity for tricyclic psychotropic drugs (37). This receptor has been designated a 5-HT$_6$ receptor and is a 437-amino-acid guanine nucleotide-binding protein-coupled receptor. It has been cloned from rat striatum, and it exhibits low (<50%) identity to previously cloned serotonin receptors. The 5-HT$_6$ receptor mRNA localizes to limbic and cortical brain regions, suggesting its possible involvement in neuropsychiatric disorders.

The newest member to date in the serotonin family is the 5-HT$_7$ receptor (38, 39). Although this receptor displays high affinity for serotonin, it is clearly distinguishable by its pharmacology from the 5-HT$_1$ receptors, and it couples positively to adenylyl cyclase. The presence of introns is also a characteristic of this receptor, which seems to be found associated with limbic brain areas.

The rich diversity of 5-HT receptor subtypes makes it important to elucidate the structure and regulation of individual receptor subtypes so that animal models for mental disorders in which serotonin is involved can be developed, and new subtype-specific drugs can be discovered and evaluated.

The regulation of 5-HT$_{1B}$ receptors has been studied utilizing 5,7-dihydroxytryptamine to destroy serotonergic neurons in rat forebrain, but results have been inconclusive or ambiguous, with reports ranging from no change to up- or down-regulation of 5-HT$_{1B}$ receptors in rat substantia nigra (40, 41). A further complication in the interpretation of chemical lesioning studies arises from the 5-HT$_{1B}$ receptor being present both as an autoreceptor and on heterologous postsynaptic elements (42).

The study of receptor regulation is greatly simplified if appropriate cell lines expressing the receptor of interest are available. Our laboratory has demonstrated the presence of 5-HT$_{1B}$ receptors in the OK (opossum kidney) cell line, an established renal proximal tubule epithelial cell line derived from the kidney of a North American opossum. We have pharmacologically characterized this receptor extensively (43) and studied its regulation (44), which has been confirmed by Unsworth and Molinoff (45).

In order to use this system to study the regulation of this important receptor subtype at the molecular level, it was necessary to clone the 5-HT$_{1B}$ receptor from the OK cell. In this study, we report the cloning, sequencing, and functional expression of an OK cell 5-HT$_{1B}$ receptor cDNA. The OK cell provided us a well characterized system from which to clone the opossum 5-HT$_{1B}$ receptor subtype. The OK cells do not express β-adrenergic receptors, which would complicate binding studies with pindolol derivatives, and the complexity of the mRNA population for G-linked receptors in the opossum kidney is greatly reduced compared to a tissue such as brain. In addition to the 5-HT$_{1B}$ receptor, OK cells also express other G-linked receptors, including the parathyroid hormone receptor (46), dopamine D$_1$ receptor (47), and the α_{2C}-adrenergic receptor (48), making it an excellent model system for studying multiple G-linked receptor regulation.

Materials and Methods

Oligonucleotides. Oligonucleotides were used as PCR and sequencing primers. Degenerate primers A and B were designed to highly conserved regions in the rat and mouse 5-HT$_{1B}$ receptors. Primer A (5′GGGAATTCGCCACCAC(G/C)CTCTC(G/C)AACGCCTTT3′) included an *Eco*RI cloning site and was situated in TM region I. Primer B (5′GGACTAGTAAATGC(C/T)CC(C/T)A(A/G)(A/G)ATGAT(G/C)CC3′) had a *Spe*I recognition sequence and was complementary to a portion of TM VI. A and B were used as both PCR and sequencing primers. The oligonucleotides for 5′ and 3′ RACE were from Life Technologies (Bethesda, MD). The gene-specific oligo(SBGSP2) for the 5′-RACE procedure (5′GGGAATTCCGTCTGTTTCAAAATCCGAGAACGAGC3′) was complementary to a portion of the third cytoplasmic loop. Other oligonucleotides used for sequencing and PCR included T3, T7, and SP6 (Promega, Madison, WI).

Probes. Primers A and B were used to generate a probe for the OK 5-HT$_{1B}$ receptor by PCR. cDNA (2 μg) were subjected to 25 cycles of PCR under standard conditions (Perkin-Elmer, Norwalk, CT), with each cycle containing a 95°, 1-min denaturation step, a 55°, 1-min annealing step, and a 72°, 3-min polymerization step. The resulting 780-bp piece was digested with *Eco*RI and *Spe*I and subcloned into pBluescript KS(+) (Stratagene, La Jolla, CA). The 780-bp cDNA was then excised using *Eco*RI and *Spe*I. [^{32}P]dCTP (ICN, Irvine, CA) was used to label this piece by the random primer method (49) using Klenow (Boerhinger-Mannheim, Indianapolis, IN). Magnagraph (Micron Separations Inc., Westboro, MA) filters were prehybridized for 1 hr at 65° in 1 M NaCl, 1% SDS, and were hybridized overnight at 65° in hybridization solution II (49). Filters were washed at 65° in 0.1× SSC and 0.1% SDS twice for 1 hr and then were autoradiographed for 12 to 24 hr at −80° with intensifying screens.

Isolation of RNA and cDNA synthesis. Total RNA was isolated from OK cells grown in Ultraculture using the guanidinium thiocyanate method (49). For the Northern blot, poly(A)$^+$ RNA was isolated from total RNA using the Fast Track mRNA isolation kit (Invitrogen, San Diego, CA). To obtain cDNA, 2 μg of total RNA were reverse-transcribed per reaction using random primers (Promega) and Superscript

reverse transcriptase (Life Technologies) according to the protocol supplied with the enzyme.

RNA analysis. Poly(A)$^+$ OK cell RNA (8 μg) was fractionated on a 1% agarose-formaldehyde gel (49) and was transferred to a Magnagraph membrane. The ^{32}P-random primer-labeled 780-bp probe was then hybridized to the filter at high stringency (42°, 50% formamide) in High Efficiency Hybridization System (Molecular Research Center Inc., Cincinnati, OH) overnight. Washes were performed at high stringency (65°, 0.1× SSC, 0.1% SDS).

Sequencing. We used both regular manual sequencing and automated sequencing, using denatured double-stranded plasmid DNA or single-stranded DNA. Sequencing was performed manually by the dideoxy chain termination method (49) using Sequenase 2.0 (United States Biochemical, Cleveland, OH). Automated sequencing was done with the *Taq* polymerase PCR method using fluorescently tagged primers or fluorescently labeled dideoxy terminators (Applied Biosystems, Inc., Foster City, CA). Sequenase reactions were also done with the fluorescent Applied Biosystems primers. The reactions were then run on an Applied Biosystems 373A automatic sequencer. Sequence data were analyzed using PC Gene.

Cloning of the complete OK 5-HT$_{1B}$ receptor. In order to acquire the missing portions of the coding region for the receptor, we used the 5′- and 3′-RACE techniques. Clones were obtained using primers and reagents from 5′- and 3′-RACE systems purchased from Life Technologies. Conditions used were as described by the supplier. For the 5′-RACE protocol, a nested gene-specific oligonucleotide designated as SBGSP2 was used.

To obtain the complete coding region of the OK 5-HT$_{1B}$ receptor, sequence information from the 5′- and 3′-RACE clones was used to design oligonucleotide primers just upstream of the ATG start site, and just downstream of the termination codon. The forward primer 5′ CCGCGGCCGCGGGCGCCATGGAACAGCCCAGCCGTCT3′ included a *Not*I site, and the reverse primer 5′GGACTAGTGAATCAGCTCGTGCGCCTGAACCGCAT3′ included a *Spe*I recognition sequence. These primers were then used to PCR the coding region of the receptor under standard buffer conditions (Perkin-Elmer). Twenty-five cycles were done on 2 μg of target cDNA (from total RNA): a 95°C, 1-min denaturing step, a 60°C, 1-min annealing step, and a 72°C, 3-min extension step. The PCR product was then digested with *Not*I and *Spe*I and subcloned into the *Not*I and *Xba*I sites of pRc/CMV (Invitrogen) and was sequenced as described above.

Cell culture and membrane preparation. CHO-K1 cells (ATCC, Bethesda, MD) and COS-1 cells were maintained in Ham's F-12 medium with 10% fetal bovine serum (Hyclone, Logan, UT). OK cells were grown as previously described (44), except that Ultraculture was used as the growth medium. All cells were grown in 95% air/5% CO_2 at 37° in a humidified atmosphere.

If either CHO or COS cells transfected with the OK cell 5-HT receptor clone were to be used for binding experiments, cells were grown in Ultraculture supplemented with L-glutamine. Membranes were prepared as previously described (44). For cyclic AMP production assays, the stably transfected CHO cells were subcultured and plated at 6×10^5 cells/well in 6-well dishes (Costar, Cambridge, MA) in Ultraculture. The cells were grown to confluency and given fresh media every 3 days.

Transfection. The entire coding region of the clone to be expressed was cloned into the *Not*I/*Xba*I sites of the eukaryotic expression vector pRc/CMV as a 1.2-kb *Not*I/*Spe*I fragment. The resulting plasmid (pRc/cmv-OKc1) was used to transiently transfect COS-1 cells by the DEAE-dextran method as previously described (49). Membrane pellets were prepared 48 hr after transfection.

In order to evaluate the functional responses of the OK 5-HT$_{1B}$ receptor and to establish permanent cell lines for regulation studies, stably transfected cell lines were produced in CHO-K1 cells using the calcium phosphate precipitation technique (50). Stable transfectants were selected with 500 μg/ml G-418 (Geneticin, GIBCO BRL, Gaithersburg, MD) and were screened by intact cell binding with [^{125}I] iodocyanopindolol (51). Briefly, replica 24-well plates (of individual clones) were washed twice with room temperature HEPES-buffered Ham's F-12 medium, and 300 μl of 0.2 nM [^{125}I]iodocyanopindolol in the same medium were added. The plates were incubated at room temperature for 1 hr and then were washed three times with room temperature HEPES-buffered Ham's F-12 medium. The monolayers then were dissolved in 0.5 ml of 0.2 N NaOH, transferred to 5-ml glass test tubes, and counted at an efficiency of 77% using a Gamma Trac 1191 gamma counter (Tracor Analytic Inc., Elk Grove, IL). Clones that showed high binding of the radioligand were chosen for further expansion. Seven independent clones were chosen with binding density (B_{max}) values ranging from 31 to 147 fmol/mg of protein as determined by membrane radioligand binding. Of these, clone B7, with a B_{max} of 147 fmol/mg of protein was selected for functional characterization.

Radioligand binding assay. To detect the expressed gene product, membrane pellets were prepared and assayed with [^{125}I]iodocyanopindolol essentially as previously described (44). In brief, a crude membrane fraction was prepared from cell pellets by homogenization and centrifugation. The pellet was stored at −80° until it was used for radioligand binding. Frozen membranes were thawed and rehomogenized in 100 to 150 volumes of 25 mM Tris-HCl, pH 8.0. Binding assays were incubated for 50 min at room temperature (~22°), in duplicate, in a final volume of 250 μl, with nonspecific binding defined by 5-HT (10 μM final concentration). When transfected COS-1 cell membranes were used, isoproterenol (3 μM final concentration) was included in the assay to mask endogenous β-adrenergic receptors. Binding assays were terminated by rapid filtration. Tubes and filters were washed 5 times within 30 sec at 22° with 6 ml of Tris-HCl, pH 8.0, containing 30 μM (±)-propranolol and 30 μM phentolamine. Protein was determined by the method of Lowry *et al.* (52) using BSA as the standard.

Cyclic AMP production assay. The [^{3}H]adenine prelabeling technique was used to assay cyclic AMP production in intact, transfected CHO cells. The cells were preincubated for 1 hr at 37° with 3 μCi [^{3}H] adenine/well (in 6-well dishes), were washed 3 times with 2.5 ml of HEPES-buffered Ham's F-12, then were incubated 2 min at 37° with 1 ml of HEPES-buffered Ham's F-12 medium containing 30 μM forskolin (to activate adenylyl cyclase) without or with 10 μM 5-HT (to inhibit adenylyl cyclase). The assays were terminated by aspiration of the medium and rapid addition of 1 ml of TCA (5% w/v). [^{3}H]ATP and cyclic [^{3}H]AMP fractions were isolated by ion exchange column chromatography (53), and the radioactivity was measured by liquid scintillation counting (Beckman Instruments, Irvine, CA).

Media, drugs, and radioligands. Cell culture media (Ham's F-12), trypsin (0.25%), and glutamine were from GIBCO. Ultraculture, a serum-free medium, was obtained from Whittaker Bioproducts (Walkersville, MD). We gratefully acknowledge Sandoz (Basel, Switzerland) for their donation of (±)-cyanopindolol and methysergide, Roussel UCLAF (Romainville, France) for RU24969, Hoffman-LaRoche (Nutley, NJ) for their gift of methiothepin, Ciba-Geigy Corp. (Summit, NJ) for providing phentolamine, and Glaxo (Middlesex, England) for their gift of sumatriptan. 8-OH-DPAT was purchased from Research Biochemicals, Inc. (Natick, MA). Serotonin, isoproterenol, and propranolol were from Sigma Chemical Co. (St. Louis, MO). All drugs except methiothepin and RU24969 were dissolved in 5 mM HCl with subsequent dilution in 5 mM HCl. Methiothepin was dissolved initially in 70% ethanol; RU24969 was dissolved initially in DMSO and subsequently diluted in 5 mM HCl. Forskolin was from Calbiochem (San Diego, CA). (−)[^{125}I]Iodocyanopindolol (2200 Ci/mmol) and [^{3}H]adenine (35 Ci/mmol) were obtained from DuPont-NEN (Boston, MA). Budget solve scintillation cocktail was purchased from Research Products International Corp. (Mount Prospect, IL).

Results

Isolation of a 5-HT$_{1B}$ clone from the OK cell line. We used primers directed at highly conserved regions in TM I and TM VI of the rat and mouse 5-HT$_{1B}$ receptors to obtain a

portion of the OK cell 5-HT$_{1B}$ receptor by PCR. The deduced amino acid sequence of this 780-bp clone was highly conserved with the rat and mouse 5-HT$_{1B}$ receptors in this region. When the 780-bp clone was labeled with [^{32}P]dCTP and used to probe a Northern blot of OK mRNA, it identified a single transcript of approximately 6 kb (Fig. 1). To complete the cloning of this receptor, we chose the RACE technique. We obtained several 5′- and 3′-RACE clones that were sequenced and shown to contain the sequence previously identified in the 780-bp clone as well as the amino and carboxy termini, respectively, of the receptor. Sequence information from the 5′- and 3′-RACE clones was then used to design primers flanking the N- and C-termini, and the complete coding region was obtained by PCR. This OK 5-HT$_{1B}$ receptor clone was designated OKc1. Sequence was confirmed by sequencing three independent PCR products. Fig. 2 shows the OKc1 receptor sequence.

The deduced amino acid sequence of OKc1 shows a high degree of amino acid identity with the mouse, rat, and human 5-HT$_{1B}$ receptors and shares several invariant structural features with the biogenic amine receptor family (Fig. 3). These features include the aspartic acid residues in TM regions II and III, and the highly conserved proline residues present in TM regions IV, V, VI, and VII. Also conserved is the DRY motif at the beginning of intracellular loop II (54). As has been reported for most G protein-coupled receptors, OKc1 appears to be a glycosylated protein. Two potential N-linked glycosylation sites exist at residues 22 and 30 in the N-terminal extracellular domain. Other consensus sequences for post-translational modifications include the following targets for protein kinases: protein kinase A potential sites at threonine residues 251 and 313, and protein kinase C at threonines 161 and 245 and at serine 236. When OKc1 is compared to other cloned 5-HT$_{1B}$ receptors, its amino acid sequence is 82% identical to the mouse, rat, and human receptors. An additional 11% of the amino acid residues are conservative substitutions. By contrast, when compared to the human 5-HT$_{1D}$ receptor, it is only 54% identical. Therefore, OKc1 likely encodes a 5-HT$_{1B}$ receptor.

Pharmacologic profile of OKc1. To determine whether OKc1 encoded a pharmacologically functional 5-HT$_{1B}$ receptor, we subcloned it into the eukaryotic expression vector pRc/CMV and transfected COS-1 cells with the recombinant plasmid pRc/CMV-OKc1. Membranes prepared from these transfected cells were then assayed using the radioiodinated β-adrenergic receptor ligand (−)[^{125}I]iodocyanopindolol (which has been shown to exhibit high affinity for 5-HT$_{1B}$ receptors). Experiments were done in the presence of 3 μM isoproterenol to mask endogenous β-adrenergic receptors. [^{125}I]Iodocyanopindolol did not show appreciable binding under these conditions to mock-transfected COS-1 cells (data not shown).

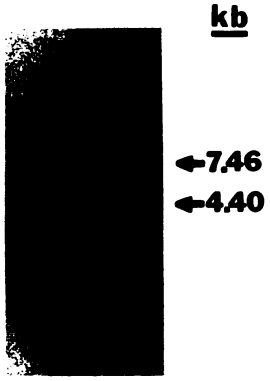

Fig. 1. A Northern blot of OK cell mRNA (8 μg) was probed with the 780-bp RT-PCR product from OK cell RNA that was labeled with [^{32}P]dCTP by the method of random priming. The blot was hybridized at 65° in High Efficiency Hybridization Solution and washed at 65° in 0.1× SSC, 0.1% SDS. A single, labeled band corresponding to approximately 6 kb was visible after a 22-hr exposure (no intensifier) to Kodak XAR film.

Membranes from cells transiently transfected with the OKc1 cDNA showed saturable, high affinity [^{125}I]iodocyanopindolol binding representing ≥95% specific binding at the concentration of the ligand equal to its equilibrium dissociation constant (K_d). Nonlinear regression analysis of the saturation data showed a single class of binding sites with an affinity (K_d) of 0.15 ± 0.02 nM (n = 3). As is customarily observed in transient transfections, the efficiency of transfection varied among individual transfections, giving B_{max} values between 398 and 779 fmol/mg of protein. The K_d value obtained for OKc1 is in good agreement with the K_d value for the 5-HT$_{1B}$ receptor in the OK cell (0.21 nM) (44).

Several competing ligands were used in inhibition binding studies to characterize pharmacologically the expressed receptor. The ligands used displayed the following rank order of potencies: cyanopindolol > RU24969 > methiothepin > serotonin > sumatriptan > methysergide > 8-OH-DPAT > isoproterenol (Table 1). This pharmacological profile agrees with that of the native OK cell receptor and is typical for the 5-HT$_{1B}$ rodent receptor, which also has a high affinity for cyanopindolol. The human 5-HT$_{1B}$ binds pindolol derivatives, but with much lower affinity (17, 55, 56). A correlation plot (Fig. 4) of the cloned OK cell 5-HT$_{1B}$ receptor pK_i values for the drugs used in Table 1 plotted against the pK_i values for those same drugs in the OK cell line gives a slope of 1.04 and a correlation coefficient (r) of 0.97, indicating a very high degree of pharmacological similarity between the two populations of receptors. Based on these drugs, the cloned 5-HT$_{1B}$ receptor is pharmacologically indistinguishable from the native OK cell receptor.

Functional assays. Stable OKc1-expressing CHO cell lines were established to generate additional cell lines for further regulation studies and to analyze the coupling of this receptor to adenylate cyclase. Mock-transfected CHO-K1 cells showed no response to either basal or forskolin-stimulated adenylyl cyclase in the presence of 10^{-4} M 5-HT (data not shown), indicating that the CHO-K1 cell does not express native cyclase-coupled serotonin receptors, including those of the 5-HT$_{1B}$ subtype. In the CHO-B7 cell line, which expresses 5-HT$_{1B}$ receptors at a density approximately double that of the OK cell, 10 μM 5-HT decreased forskolin-stimulated cyclic AMP production (Fig. 5). Thus, the OKc1 cDNA encodes a functional 5-HT$_{1B}$ receptor.

Discussion

Until recently, the 5-HT$_{1B}$ subtype was thought to be present only in the rat, mouse, and opossum, whereas the 5-HT$_{1D}$ receptor had been found in the guinea pig, pig, calf, and human. Several groups (16–20) have now shown that a species orthologue of the rodent 5-HT$_{1B}$ receptor does exist in the human. Its pharmacological profile, however, differs somewhat from that of the rodent 5-HT$_{1B}$ receptors, notably in its lower affinity for β-adrenergic ligands such as pindolol, and for several serotonergic compounds, including RU24969 (17, 55, 56).

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ATGGAACAGCCCAGCCGTCTGTGCTCTCCGCCAGCCTCCGGTTCCCTGACCTCCTCGCAG   60
METGluGlnProSerArgLeuCysSerProProAlaSerGlySerLeuThrSerSerGln   20

ACTAATCATTCTACCTTCCCGAATCCCAACTGCAGCGCTCCGGATCTGGAGCCATACCAG  120
ThrAsnHisSerThrPheProAsnProAsnCysSerAlaProAspLeuGluProTyrGln   40
   Δ                  Δ

GACTCGATTGCACTCCCTTGGAAGGTGCTTCTGGCCACGTTCCTTGGACTCATCACCTTG  180
AspSerIleAlaLeuProTrpLysValLeuLeuAlaThrPheLeuGlyLeuIleThrLeu   60

GGCACCACGCTCTCGAACGCCTTTGTGATCGCCACTGTCTCTCGGACTAGGAAGCTGCAC  240
GlyThrThrLeuSerAsnAlaPheValIleAlaThrValSerArgThrArgLysLeuHis   80
                                          ▲

ACTCCTGCCAACTACCTGATCGCCTCCCTGGCAGTGACTGACTTGCTTGTGTCTATCCTG  300
ThrProAlaAsnTyrLeuIleAlaSerLeuAlaValThrAspLeuLeuValSerIleLeu  100

GTGATGCCCATCAGCACTATGTACACGGTCACCGGCAGGTGGACTCTGGGCCAGGTTGTC  360
ValMetProIleSerThrMetTyrThrValThrGlyArgTrpThrLeuGlyGlnValVal  120
                          ▲

TGTGATTTCTGGCTGTCCTCGGACATTACCTGTTGCACAGCTTCCATACTGCATCTCTGT  420
CysAspPheTrpLeuSerSerAspIleThrCysCysThrAlaSerIleLeuHisLeuCys  140

GTTATCGCTCTGGACCGCTACTGGGCCATTACAGACGCGGTAGAGTACTCCGCTAAAAGG  480
ValIleAlaLeuAspArgTyrTrpAlaIleThrAspAlaValGluTyrSerAlaLysArg  160
                                    ▲

ACTCCCAAGCGAGCAGCTGGAATGATTATTATGGTATGGGTCTTCTCTGTATCCATTTCA  540
ThrProLysArgAlaAlaGlyMetIleIleMetValTrpValPheSerValSerIleSer  180
▲

ATGCCCCCACTTTTCTGGCGCCAGGCCAAGGCCGAGGAGGTGGCAGACTGCTCAGTGAAC  600
MetProProLeuPheTrpArgGlnAlaLysAlaGluGluValAlaAspCysSerValAsn  200

ACAGACCACATTCTCTATACAGTCTACTCTACTGTGGGCGCCTTCTACTTCCCCACCCTG  660
ThrAspHisIleLeuTyrThrValTyrSerThrValGlyAlaPheTyrPheProThrLeu  220

CTGCTTATTGCCCTTTATGGCCGCATCTATGTGGAAGCTCGTTCTCGGATTTTGAAACAG  720
LeuLeuIleAlaLeuTyrGlyArgIleTyrValGluAlaArgSerArgIleLeuLysGln  240

ACGCCCAACAGGACGGGCAAACGTCTAACTCGTGCTCAACTGATCACCGACTCCCCAGGA  780
ThrProAsnArgThrGlyLysArgLeuThrArgAlaGlnLeuIleThrAspSerProGly  260
          ▲              ●

TCCTCTTCCTCGGGCACCTCCATTAACTCGAGAGCCCCCGAGGGACCCAGTGAATCCGGG  840
SerSerSerSerGlyThrSerIleAsnSerArgAlaProGluGlyProSerGluSerGly  280

TCCCCAGTGTATGTGAACCAAGTAAAGGTGAAAGTCTCTGACGCTCTCCTGGAAAAGAAG  900
SerProValTyrValAsnGlnValLysValLysValSerAspAlaLeuLeuGluLysLys  300

AAGCTCATGGCCGCTAGGGAGCGAAAAGCCACCAGAACGCTGGGGATCATTTTAGGAGCC  960
LysLeuMetAlaAlaArgGluArgLysAlaThrArgThrLeuGlyIleIleLeuGlyAla  320
                    ●

TTCATCGTCTGCTGGCTGCCTTTCTTTATCATCTCCCTGGCATTACCTATCTGTGATGAC 1020
PheIleValCysTrpLeuProPhePheIleIleSerLeuAlaLeuProIleCysAspAsp  340

GCCTGCTGGTTCCACCTGGCCATCTTTGACTTCTTTAATTGGCTAGGCTATCTCAACTCC 1080
AlaCysTrpPheHisLeuAlaIlePheAspPhePheAsnTrpLeuGlyTyrLeuAsnSer  360

CTCATTAACCCCATCATCTATACCAAGTCCAATGATGACTTCAAACAAGCTTTCCAAAAA 1140
LeuIleAsnProIleIleTyrThrLysSerAsnAspAspPheLysGlnAlaPheGlnLys  380

CTGATGCGGTTCAGGCGCACGAGCTGA 1167
LeuMetArgPheArgArgThrSer---  388
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Fig. 2. The nucleotide and deduced amino acid sequence of OKc1: (Δ), postulated N-linked glycosylation sites. The seven predicted transmembrane regions are *overlined*. (▲), (●), putative sites for phosphorylation by protein kinase C and protein kinase A, respectively.

We have shown previously the existence of a 5-HT receptor in the OK cell line that exhibits the pharmacology of a 5-HT_{1B} receptor (57). Here, we report the cloning and characterization of the 5-HT_{1B} receptor from these cells. The deduced amino acid sequence of the receptor described here is quite similar (82% identical and 11% conservative substitutions) to the previously cloned 5-HT_{1B} receptors (13, 15–20). As observed for the other G protein-coupled receptors, the putative TM regions from the 5-HT_{1B} receptors of various species have a high degree of sequence identity, with mostly conservative substitutions occurring where differences are present. TM regions II, III, and V have 100% amino acid identity. The amino terminus has the least amino acid sequence identity (44%); the third cytoplasmic loop is 92% identical, whereas in the carboxy terminus, 16 out of 18 amino acid residues are identical. It is interesting to note that there are several instances in which the opossum receptor expresses the same amino acid residue as the human 5-HT_{1B} receptor, instead of the rat or mouse residue

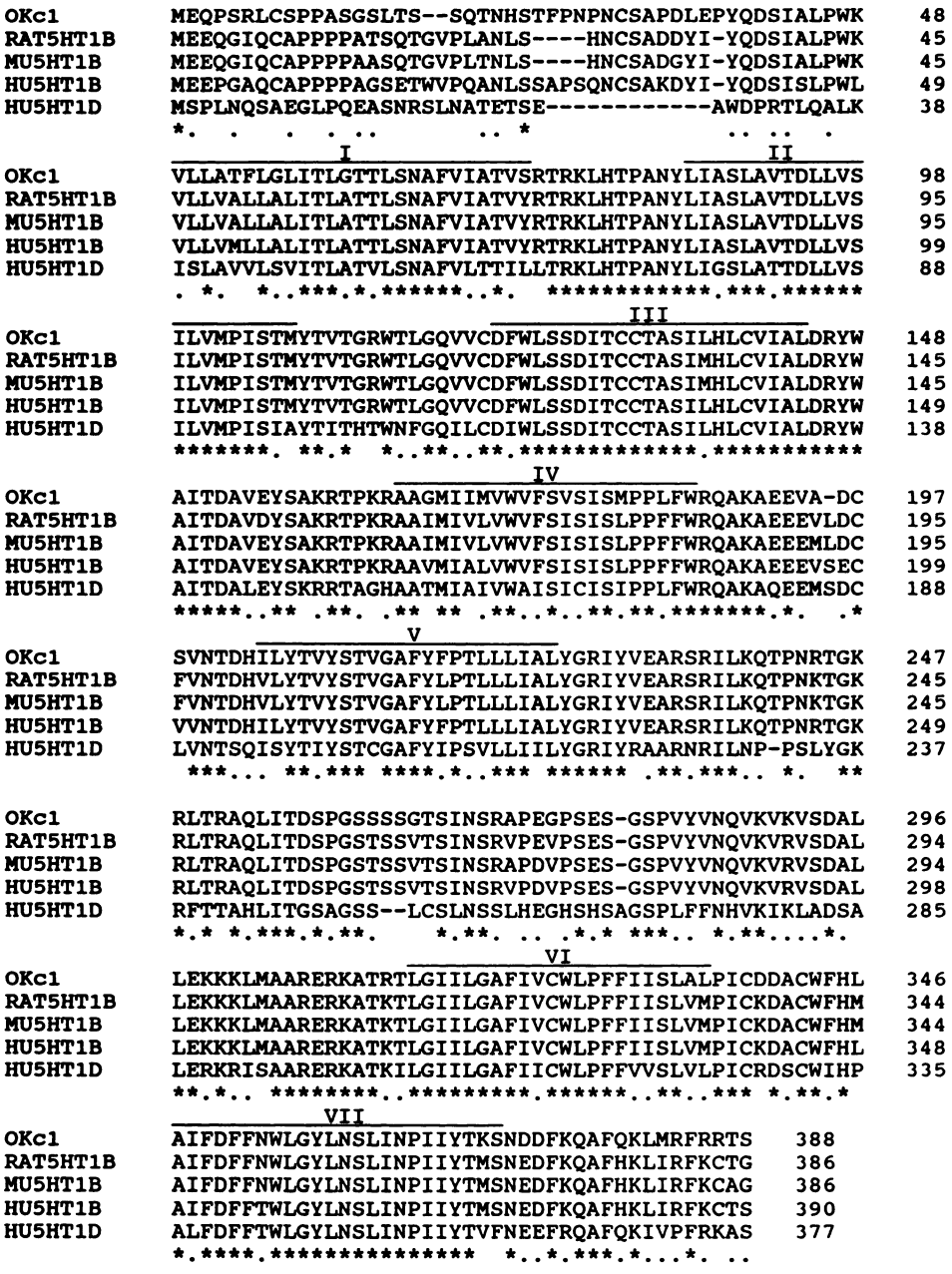

Fig. 3. Alignment of the deduced amino acid sequence of OKc1 with the rat, mouse and human 5-HT$_{1B}$ receptors and the human 5-HT$_{1D}$ receptor. The sequences of the rat 5-HT$_{1B}$, mouse 5-HT$_{1B}$, human 5-HT$_{1B}$, and human 5-HT$_{1D}$ receptors were from Refs. 13, 15, 16, and 23, respectively. An *asterisk* indicates identical residues; well conserved amino acids are indicated by a *dot*. Transmembrane regions are *overlined*. The amino acid sequences were aligned using CLUSTAL (67).

(Ile 204, Phe 217, Arg 244, Leu 346, and Ser 388), whereas in other instances (Ala 52, Leu 184, Gly 275, Lys 291, Leu 335, Gln 379, and Arg 385), it agrees with the human 5-HT$_{1D}$ receptor.

The recent cloning of the human parathyroid hormone receptor (58) provides another human G-linked receptor to compare with its opossum counterpart (46). When the human and rat (59) parathyroid receptor amino acid sequences are compared, they are 90% identical, versus the human and opossum receptors, which share only 78% identity. The main divergences between the human and opossum parathyroid receptor sequences occur near the amino terminus, between transmembrane regions II and III, and at the carboxy terminus. This is very similar to the degree of amino acid identity (82%) observed between OKc1 and the other cloned 5-HT$_{1B}$ receptors (13, 15–20).

As expected, the opossum clone has the rodent Asn (353) instead of the human Thr residue in TM VII, which presumably confers upon it the ability to bind pindolol and its derivatives (55, 56, 60). It also has a relatively large third cytoplasmic loop (88 amino acids) and a short carboxy terminus (18 amino acids), which are features associated with receptors that couple to the inhibition of adenylyl cyclase. This is consistent with the 5-HT inhibition of cyclic AMP production that we observed in CHO cells stably transfected with OKc1 cDNA. The OK cell 5-HT$_{1B}$ receptor has been shown previously to inhibit cyclic AMP production (57). The clone also has a positively charged lysine (380) residue in the carboxy terminus, as do other receptors that couple negatively to adenylyl cyclase. This position has a negatively charged residue in receptors that activate adenylyl cyclase, suggesting that this amino acid plays a role in the interaction between the receptor and G proteins (61).

TABLE 1

Drug affinities for the cloned OK 5-HT$_{1B}$ receptor

Apparent inhibition constant (K_i) values of β-adrenergic and serotonergic ligands for the cloned OK 5-HT$_{1B}$ receptor were determined from inhibition experiments. Inhibiton experiments, routinely consisting of 11 concentrations of the unlabeled ligand, were analyzed using nonlinear least squares parametric curve-fitting programs to obtain IC_{50} values. IC_{50} values were converted to K_i values by the method of Cheng and Prusoff (68) and are presented as geometric means ± standard error. If $n = 2$, then the two values are given. For comparison, previously published K_i values for the native OK cell receptor are also listed (57). Values for sumatriptan were generated for OKc1 in parallel with OK cell membranes for this study.

Drug name	COS-OKc1 Cells					OK Cells			
	K_i	SE	pK_i	n	Slope	K_i	pK_i	n	Slope
Cyanopindolol	0.75	0.08	9.12	4	1.07	0.73	9.14		
RU24969	1.22	0.14	8.91	3	0.82	1.68	8.77		
Methiothepin	4.95	0.10	8.31	3	0.72	8.25	8.08		
Serotonin	41.4	1.5	7.38	3	0.90	60	7.22		
Methysergide	217	23	6.66	3	0.78	945	6.02		
8-OH-DPAT	7,470	1,290	5.13	3	0.59	6,337	5.20		
Sumatriptan	100	24	7.00	4	0.87	37; 41	7.41	2	0.68
Isoproterenolol	23,800; 24,600		4.62	2	1.24				

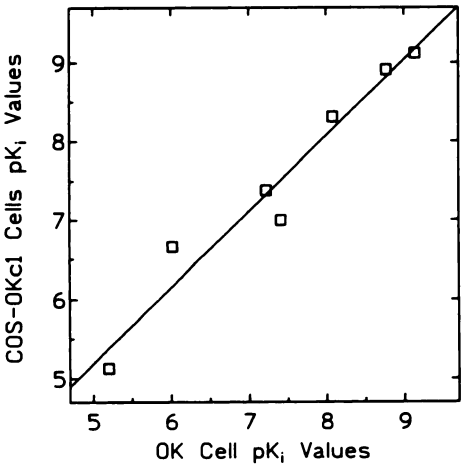

Fig. 4. Correlations between affinity constant (pK_i values) of different β-adrenergic and serotonergic ligands for OKc1 and the pharmacologically defined OK cell native receptor. Values correspond to drugs listed in Table 1. $r = 0.97$.

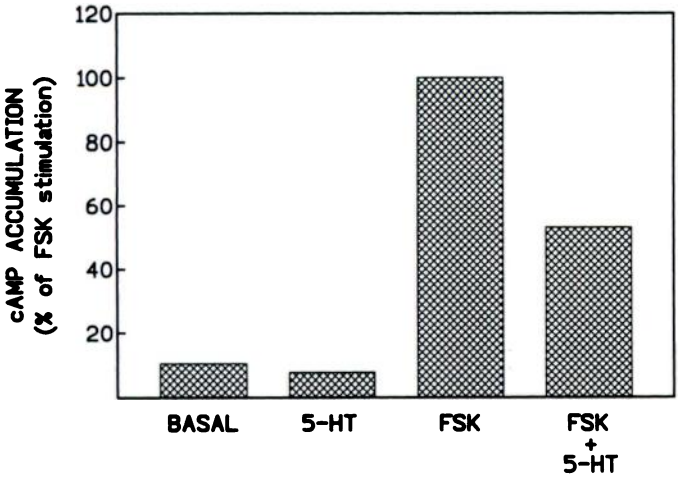

Fig. 5. 5-HT inhibition of cyclic AMP accumulation in CHO cells stably expressing OKc1. Cyclic AMP measurements were performed using intact cells as described in Materials and Methods. Forskolin (FSK) was used at a concentration of 30 μM and 5-HT at 10 μM. Treatments are indicated below each *bar* (basal value = no drug or FSK). Data represent mean values from triplicate determinations derived from a representative experiment, which was repeated 3 times.

The North American opossum (order Didelphimorphia, species *Didelphis virginiana*) is thought to have diverged from the Australian marsupials in the early to mid-Cretaceous period, 103 to 128 million years ago (62), which is about the same time as the marsupials and placental mammals are believed to have diverged from their last common ancestor. The order Rodentia (rodents) is thought to have diverged from mammals much more recently, in the late Mesozoic to early Cenozoic period, about 50 to 65 million years ago (63). Despite the evolutionary time scale separating the placental mammals and the marsupials, the size of the 5-HT$_{1B}$ mRNA in the OK cell line (6 kb) is approximately the same as that of the mouse 5-HT$_{1B}$ mRNA in brain and spinal cord (15). The conservation of message size and consensus sites for post-translational modification, together with the high level of amino acid identity among the opossum, rat, mouse, and human 5-HT$_{1B}$ receptors, argues for a strongly conserved structure/function relationship.

Hamel *et al.* (64) have recently studied the localization of the 5-HT$_{1B}$ receptor message in both human and bovine cerebral arteries, vessels that presumably are involved in migraine pathophysiology. They were unable to find the presence of either 5-HT$_{1B}$ or 5-HT$_{1D}$ transcripts in intraparenchymal microvessels or capillaries isolated from bovine or human cerebral cortex. If the effects of sumatriptan are primarily vascular, they most likely involve cerebral blood vessels at the surface of the brain. Their study points to the 5-HT$_{1B}$ subtype as the main putative target for migraine drug therapies.

The OK cell appears to be a reasonable model of the CNS 5-HT$_{1B}$ receptor. Ciaranello *et al.* (65) have shown that the 5-HT$_{1B}$ receptor in the mouse kidney and brain are very similar, if not identical, in their properties. This finding has been confirmed and extended by Matsumoto *et al.* (66), who studied 5-HT$_{1B}$ sites in rat spinal cord by [^{125}I]iodocyanopindolol binding. The pharmacology of this receptor also appeared to be identical to the 5-HT$_{1B}$ site identified by [^{125}I]iodocyanopindolol in brain and peripheral tissues. The advantages of the OK cell line include: 1) it appears to be the only cell line in continuous culture that naturally expresses a well characterized 5-HT$_{1B}$ receptor subtype; 2) it does not express other 5-HT receptors that would complicate binding and functional assays; 3) OK cells do not express β-adrenergic receptors that would interfere with radioligand binding assays when using [^{125}I] iodocyanopindolol; and 4) the genetic regulation of receptor mRNA and receptor expression can be studied directly without

interference from endogenous neurotransmitters and neuronal pathways. Additionally, the stable expression of this cloned receptor in other cell lines now will provide the tools to further elucidate the molecular biology of the 5-HT$_{1B}$ receptor. This makes the OK cell an attractive model for the study of the regulation of this important receptor subtype.

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