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RAPID COMMUNICATIONS

CONVERSION OF THE HUMAN 5-HT$_{1D\beta}$ SEROTONIN RECEPTOR TO THE RAT 5-HT$_{1B}$ LIGAND-BINDING PHENOTYPE BY THR^{355}ASN SITE DIRECTED MUTAGENESIS

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ABSTRACT — The human 5-HT$_{1D\beta}$ serotonin receptor and its rat homolog (also called the 5-HT$_{1B}$ receptor) share 93% amino acid identity, yet display markedly different pharmacological specificities. Comparison of deduced amino acid sequences among these and other recently cloned receptors suggested that this phenotypic difference might be attributable to a single human threonine^{355}/rat asparagine^{351} amino acid difference in the putative seventh membrane spanning regions. We now report that Thr^{355}Asn mutagenesis of the human 5-HT$_{1D\beta}$ receptor alters the binding characteristics of the recombinant receptor in [^{3}H]5-HT binding assays to a profile very similar to that of the rat 5-HT$_{1B}$ binding site. These results confirm that this single amino acid difference is responsible for the majority of the known pharmacological discrepancies between human and rat observed for 5-HT$_{1D\beta}$ (5-HT$_{1B}$) receptors.

Recent months have seen an explosion in the number of receptors for serotonin (5-hydroxytryptamine, 5-HT) that have been cloned. We have cloned two human 5-HT$_{1D}$ receptors, members of the seven α-helix G protein linked receptor family, as well as their rat homologs [1-3], and similar results have been reported independently by others [4-10]. Pharmacological characterization of these gene products has shown that one such 5-HT$_{1D}$ receptor subtype, also referred to as the 5-HT$_{1D\alpha}$ receptor, shows nearly identical pharmacological profiles in human and rat [3]. The other currently known 5-HT$_{1D}$ receptor shows a classical 5-HT$_{1D}$-like receptor pharmacological profile in human, but a strikingly different, 5-HT$_{1B}$-like profile in rat [3,8,9] and mouse [10]. The 5-HT$_{1B}$ phenotype is distinguished from the classic 5-HT$_{1D}$ profile chiefly by its much higher affinity for pindolol and its derivatives and somewhat higher affinity for the indole RU 24969. Consequently, this second human 5-HT$_{1D}$ receptor has been referred to as either the 5-HT$_{1D\beta}$ receptor [6,7] or the human 5-HT$_{1B}$ receptor [2,4].

The unusual degree of species-specific pharmacological difference for the 5-HT$_{1D\beta}$ receptor was not entirely unexpected, based on observations from animal tissues and cells (reviewed in [11]). Nearly identical 5-HT$_{1B}$-like phenotypes have been observed in some rodents and the evolutionarily quite distantly related American Opossum, but no receptors with intermediate 5-HT$_{1B}$/5-HT$_{1D}$-hybrid phenotypes have been found in other mammalian species. These observations suggested that the species-specific differences in the 5-HT$_{1B}$/5-HT$_{1D}$ receptor family might be due to a single amino acid difference, or perhaps at most a very few. Just where such a difference might lie was suggested by the work of Kobilka and colleagues on other G protein coupled receptors [12]. In a study of chimeric adrenergic receptors, high affinity for [^{125}I]cyanopindolol, a key characteristic of β-adrenergic receptors as well as 5-HT$_{1B}$ receptors, was found to be conferred by the predicted seventh membrane spanning region. More recent site directed mutagenesis studies have shown that a single critical amino acid position, corresponding to Thr^{355} in the human 5-HT$_{1D\beta}$ receptor, greatly influences the drug-binding profile of adrenergic [13] as well as 5-HT$_{1A}$ serotonergic receptors [14]. The presence of an asparagine in this position correlates with high pindolol affinity.

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We, as well as Adham and co-workers, noted that the cloned rat 5-HT_{1B} receptor also has a predicted asparagine residue in this critical position in the predicted seventh hydrophobic region, while its human homolog, the 5-$HT_{1D\beta}$ receptor, has a threonine in this position [2,8], as do the human [1] and rat [3] 5-$HT_{1D\alpha}$ receptors. We wished to test directly the hypothesis that this single amino acid difference provides much of the pharmacological distinction between rat and human 5-$HT_{1D\beta}$ (5-HT_{1B}) receptors. In this paper we describe the pharmacological analysis of a $Thr^{355}Asn$ mutant of the human 5-$HT_{1D\beta}$ receptor. We show that this mutated receptor is altered to the classic rat 5-HT_{1B}-like (high cyanopindolol-affinity) phenotype.

MATERIALS AND METHODS

Construction of wild type and $Thr^{355}Asn$ mutant human 5-$HT_{1D\beta}$ receptor constructs for expression. A 528 bp Xma I fragment of the human 5-$HT_{1D\beta}$ (5-HT_{1B}) receptor [2] which encoded the presumed seventh membrane spanning region was subcloned into pBluescript II KS(-) in the T3→T7 orientation. This served as a template for mutation using the polymerase chain reaction-based method of Ito and co-workers [15]. The mutant primer employed was 5'-ATAGCCCAGCCAATTGAAGAAGTCAAAG-3'. Both strands of the mutated Xma I fragment were then sequenced to ensure that no inadvertent mutations had been introduced. The mutated Xma I fragment was then excised and ligated into the adjacent 1.3 kb Hind III-Xma I fragment of the receptor gene, also in pBluescript II KS(-) in the T3→T7 orientation, to recreate the entire, now mutated open reading frame. A Xho I-Bam HI fragment containing the open reading frame was then subcloned into the eukaryotic expression vector pSVL (Pharmacia), in the proper orientation. A corresponding wild type Bam HI fragment of the unaltered gene was also subcloned into pSVL for wild type receptor expression.

Receptor expression and [^{3}H]5-HT binding assays. Wild type or mutant expression construct DNA was used to transfect COS-7 cells (20 μg/150 mm culture plate) using the method described in Ref. 16. After 48-72 hr, the cells were harvested by scraping into Dulbecco's phosphate-buffered saline supplemented with 5 mM EDTA and assessed for [^{3}H]5-HT (24-29 Ci/mmol, New England Nuclear) binding as previously described [3]. Sources of drugs were as described in Ref. 1. IC_{50} values were determined by the method of Parker and Waud [17].

RESULTS AND DISCUSSION

We constructed a human 5-$HT_{1D\beta}$ $threonine^{355}$asparagine mutant to see if this change would explain the great interspecies pharmacological differences found for this receptor. The affinities of drugs examined for the wild type human 5-$HT_{1D\beta}$ receptor expressed in COS-7 cells as assessed in [^{3}H]5-HT competition binding assays (Table 1) were in good agreement with values that we previously obtained using stably transfected HeLa cells [2]. Introduction of the $Thr^{355}Asn$ mutation had no discernable effect on the affinity of 5-HT. However, the mutation increased the affinity of the receptor for (±)cyanopindolol by 180-fold and for RU 24969 by 30-fold (Table 1), placing the affinities of these two compounds in the range of what would be expected of a rat 5-HT_{1B} receptor. Although not generally regarded as a defining difference between 5-HT_{1D} and 5-HT_{1B} receptor profiles, studies of

Table 1. Effect of the $Thr^{355}Asn$ mutation on drug affinity for [^{3}H]5-HT (2 nM) labeled 5-$HT_{1D\beta}$ receptors expressed in COS-7 cells

Drug	Human 5-$HT_{1D\beta}$ wild type IC_{50} (nM)	Human 5-$HT_{1D\beta}$ $Thr^{355}Asn$ mutant IC_{50} (nM)	Mutation-induced shift (fold)
(±)Cyanopindolol	150 ± 30	0.84 ± 0.28	180
RU 24969	97 ± 19	3.3 ± 0.2	30
5-HT	5.5 ± 2.3	5.5 ± 0.1	1.0
Sumatriptan	14 ± 4	190 ± 30	(13)
CGS 12066B	2.1 ± 0.6	30 ± 9	(14)
Yohimbine	63 ± 13	2,000 ± 300	(31)
8-OH-DPAT	320 ± 20	18,000 ± 1,600	(56)

IC_{50} values are expressed as means ± SEM for N = 3.

brain tissues in the appropriate species have generally found (±)-8-hydroxy-N,N-dipropyl-2-aminotetralin (8-OH-DPAT) to have substantially greater affinity for 5-HT_{1D} type brain binding sites [18-20] than 5-HT_{1B} sites [21]. As would be predicted by these differences, the $Thr^{355}Asn$ mutation decreased the affinity of the recombinant human 5-$HT_{1D\beta}$ receptor for 8-OH-DPAT by 56-fold. Indeed, brain membrane 5-HT_{1D} binding assays have generally been performed in the presence of 100 nM 8-OH-DPAT, a practice which may decrease the apparent affinity of the human 5-$HT_{1D\beta}$ receptor for this compound and minimize the apparent human versus rat 5-$HT_{1D\beta}$ selectivity for 8-OH-DPAT. Interestingly, an $Asn^{385}Val$ mutation in the corresponding position of the 5-HT_{1A} receptor had little effect on the affinity of this receptor for 8-OH-DPAT [14]. Introduction of the $Thr^{355}Asn$ mutation also decreased the affinity of the 5-$HT_{1D\beta}$ receptor for yohimbine. This alteration is in keeping with the observations of Suryanarayana and co-workers, who also found a decreased yohimbine affinity for a human α_2-adrenergic receptor with the corresponding $Phe^{412}Asn$ mutation [13]. Affinities for the anti-migraine drug sumatriptan and CGS 12066B were also decreased for the mutant human 5-$HT_{1D\beta}$ receptor.

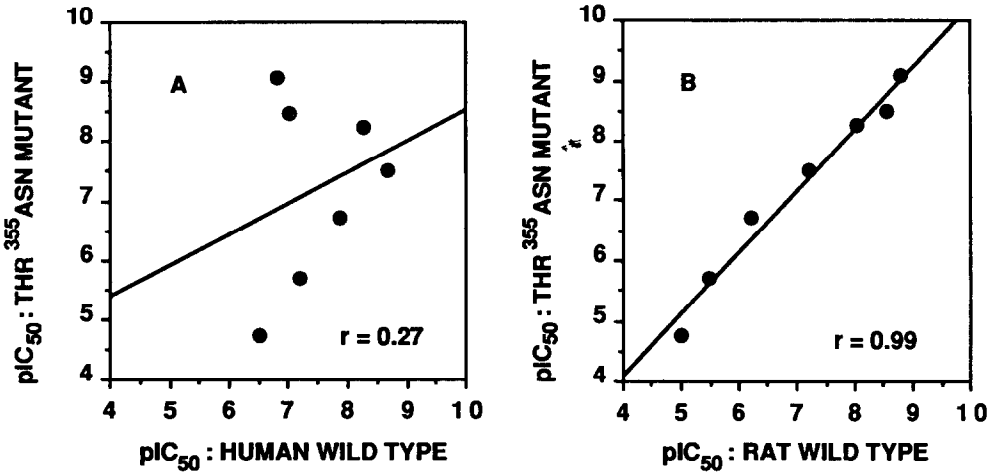

Fig. 1. Correlation between observed pIC_{50} values of drugs competing for [^{3}H]5-HT binding to $Thr^{355}Asn$ mutant human 5-$HT_{1D\beta}$ receptors and (**A**): wild type human 5-$HT_{1D\beta}$ receptors and (**B**): wild type rat 5-$HT_{1D\beta}$ (5-HT_{1B}) receptors. Rat IC_{50} values are from Ref. 3. The IC_{50} for 8-OH-DPAT at the rat receptor was determined to be >10 μM, and so this value was used for plotting.

The mutation-induced changes in drug affinity were emphasized by the poor correlation of the pIC_{50} values for the mutant receptor with the values for the wild type receptor (Fig. 1A). In contrast, there was a high correlation of the pIC_{50} values of the mutant human receptor with those of the rat 5-HT_{1B} receptor (Fig. 1B). These results confirm that the residue occupying the mutated position is a key determinant of serotonin receptor pharmacological selectivity, but they do not rule out the possibility that other amino acid residue differences may also contribute to the inter-species pharmacological difference observed in a more minor way. Data such as these in combination with molecular modeling may eventually shed light on the physical nature of ligand/receptor binding.

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